EXHIBIT 4

US006831994B2

(12) **United States Patent**
Bridgham et al.

(10) **Patent No.: US 6,831,994 B2**
(45) **Date of Patent: Dec. 14, 2004**

(54) **SYSTEM AND APPARATUS FOR SEQUENTIAL PROCESSING OF ANALYTES**

(75) Inventors: **John Bridgham**, Hillsborough, CA (US); **Kevin Corcoran**, Fremont, CA (US); **George Golda**, El Granada, CA (US); **Michael C. Pallas**, San Bruno, CA (US); **Sydney Brenner**, La Jolla, CA (US)

(73) Assignee: **Lynx Therapeutics, inc.**, Hayward, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 196 days.

(21) Appl. No.: **09/908,130**

(22) Filed: **Jul. 17, 2001**

(65) **Prior Publication Data**

US 2002/0051992 A1 May 2, 2002

Related U.S. Application Data

(62) Division of application No. 09/424,028, filed as application No. PCT/US98/11224 on May 22, 1998, now Pat. No. 6,406,848, which is a continuation of application No. 08/862,610, filed on May 23, 1997, now abandoned.

(51) **Int. Cl.**[7] **G06K 9/00**

(52) **U.S. Cl.** **382/133**; 250/461.2

(58) **Field of Search** 382/100, 128, 382/129–134, 181, 278; 250/458.1, 461.2; 356/317, 318; 359/225, 368; 422/131, 165; 435/6, 69.1; 436/518; 514/12

(56) **References Cited**

U.S. PATENT DOCUMENTS

3,413,464 A	11/1968	Kamentsky	250/304
4,125,828 A	11/1978	Resnik	382/134
4,354,114 A	10/1982	Karnaukhov	250/458.1
4,661,225 A	4/1987	Penniman	204/549
4,911,782 A	3/1990	Brown	216/33

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

EP	0 392 546	10/1990
EP	0 573 098	12/1993
WO	WO96/12014 A1	4/1996

OTHER PUBLICATIONS

Ekins and Chu, "Multianalyte microspot immunoassay–Microanalytical 'compact disk' of the future", Clinical Chemistry, 37(11): 1955–1967, 1991.

Lam and Lebl, "Selectide Technology: Bead–binding Screening", Methods: A companion to Methods in Enzymology, 6:372–380, 1994.

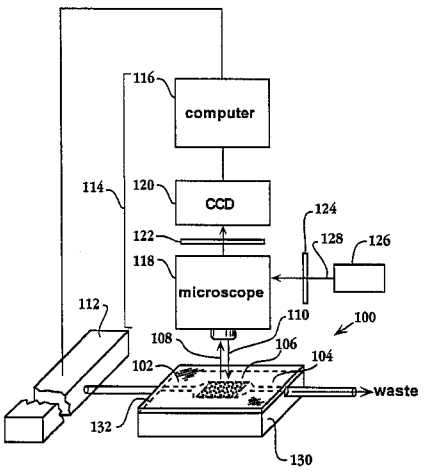
Hiraoka et al., "The use of a charge–coupled device for quantitative optical microscopy of biological structures", Science, 238: 36–41, 1987.

Primary Examiner—Jayanti K. Patel
(74) *Attorney, Agent, or Firm*—Vincent M. Powers; Lee Ann Gorthey; Perkins Coie LLP

(57) **ABSTRACT**

An apparatus and system are provided for simultaneously analyzing a plurality of analytes anchored to microparticles. Microparticles each having a uniform population of a single kind of analyte attached are disposed as a substantially immobilized planar array inside of a flow chamber where steps of an analytical process are carried out by delivering a sequence of processing reagents to the microparticles by a fluidic system under microprocessor control. In response to such process steps, an optical signal is generated at the surface of each microparticle which is characteristic of the interaction between the analyte carried by the microparticle and the delivered processing reagent. The plurality of analytes are simultaneously analyzed by collecting and recording images of the optical signals generated by all the microparticles in the planar array. A key feature of the invention is the correlation of the sequence of optical signals generated by each microparticle in the planar array during the analytical process.

4 Claims, 10 Drawing Sheets



U.S. PATENT DOCUMENTS

5,116,765	A		5/1992	Watanabe 436/165
RE34,214	E		4/1993	Carlsson
5,235,522	A	*	8/1993	Bacus 702/19
5,479,252	A	*	12/1995	Worster et al. 356/237.5
5,578,832	A		11/1996	Trulson 250/458.1
5,604,097	A		2/1997	Brenner 435/6
5,631,734	A		5/1997	Stern 356/317
5,705,402	A		1/1998	Leland 436/526
5,707,799	A		1/1998	Hansmann 435/6
5,741,647	A		4/1998	Tam 435/6
5,800,992	A		9/1998	Fodor 435/6
5,834,195	A		11/1998	Benkovic 435/6
5,854,684	A		12/1998	Stabile 356/440
5,856,174	A		1/1999	Lipshutz 435/286.5
5,872,623	A		2/1999	Stabile 356/73
5,985,214	A	*	11/1999	Stylli et al. 422/65
6,023,540	A		2/2000	Walt 385/12
6,054,277	A	*	4/2000	Furcht et al. 435/6
6,327,377	B1	*	12/2001	Rutenberg et al. 382/133

* cited by examiner

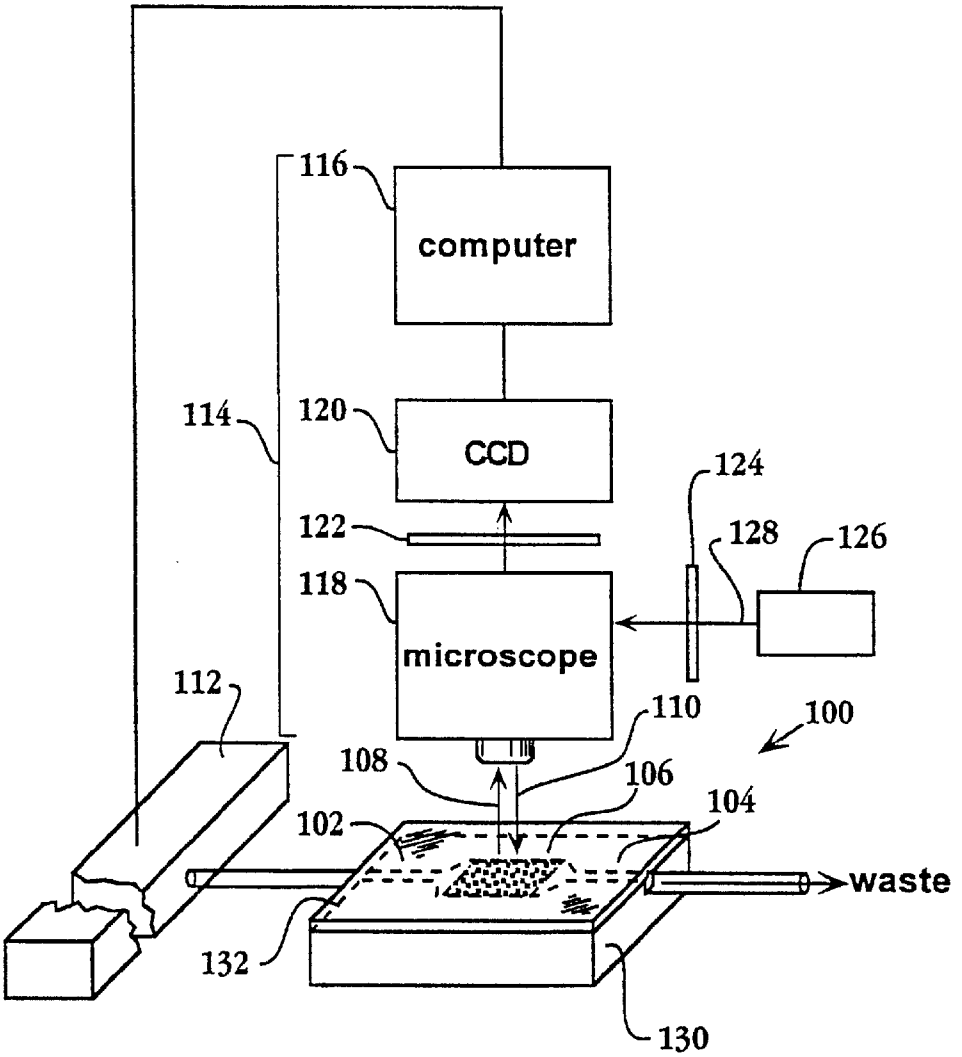

116
computer
114
120
CCD
124
122
128
126
118
microscope
112
110
100
108
106
104
102
waste
132
130

Fig. 1A

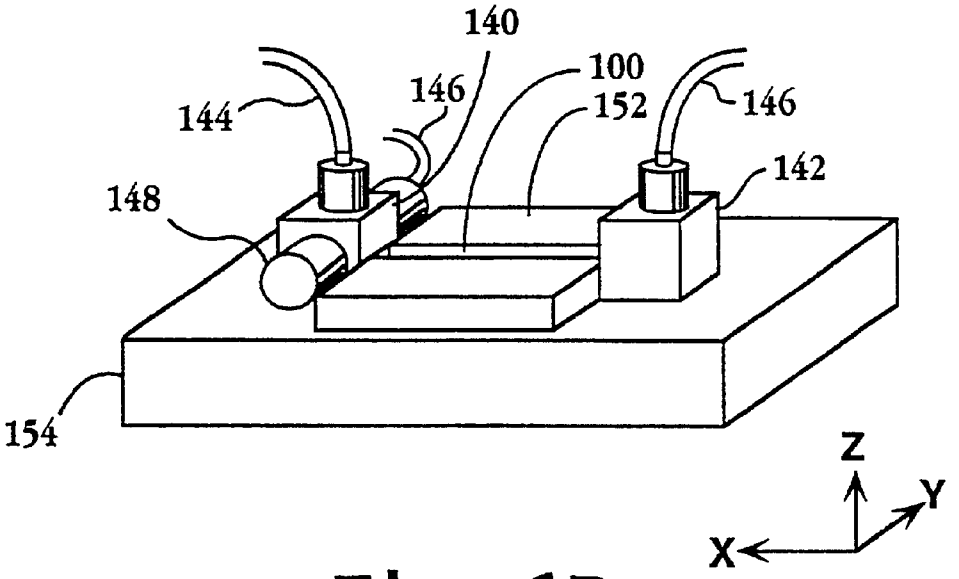


Fig. 1B

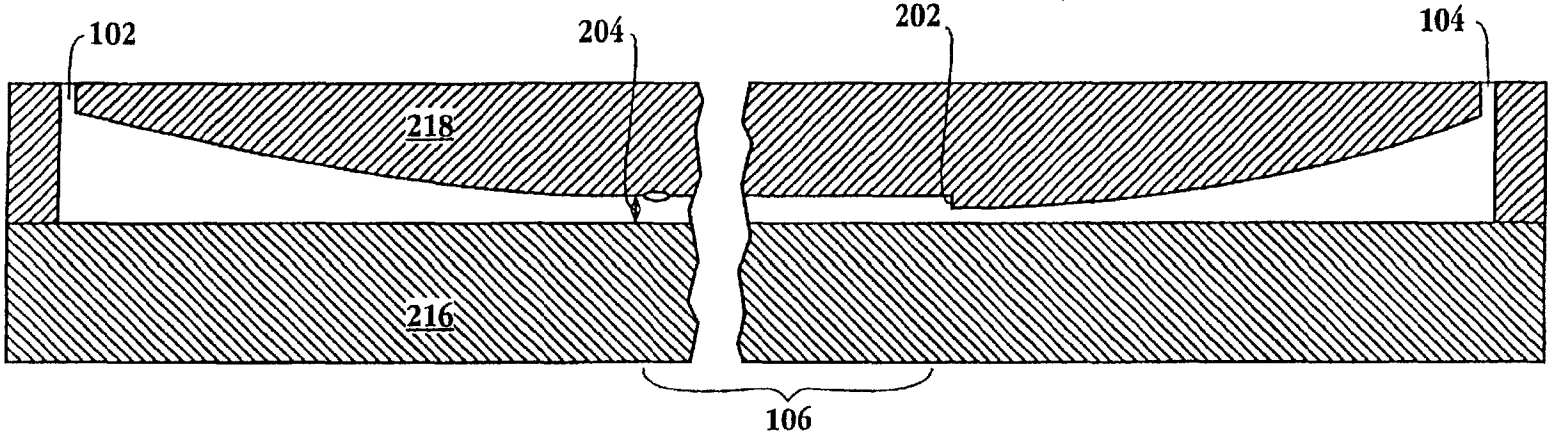

102
204
202
104
218
216
106

Fig. 2A

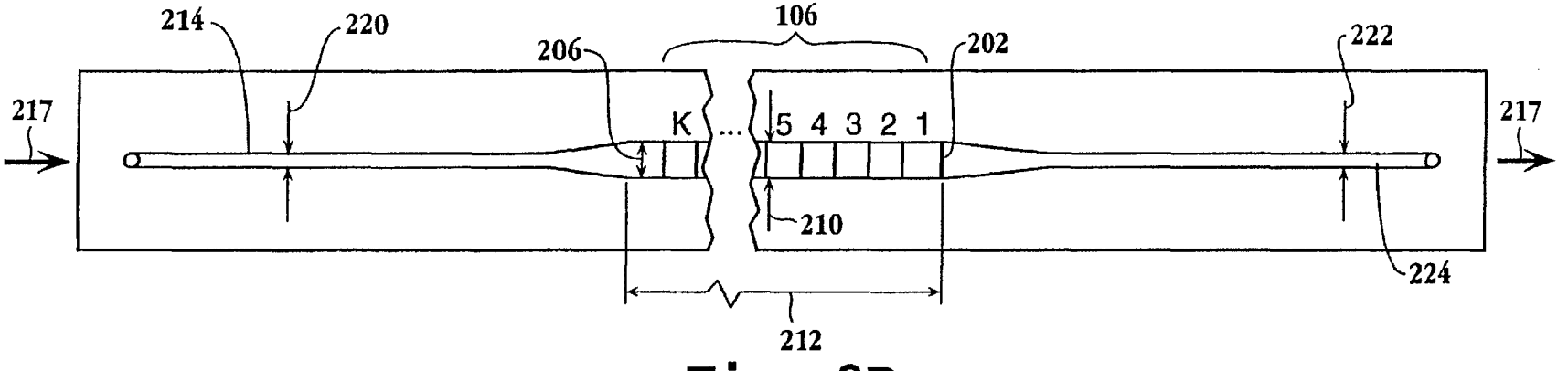

214
220
206
106
202
222
217
K
...
5 4 3 2 1
217
210
224
212

Fig. 2B

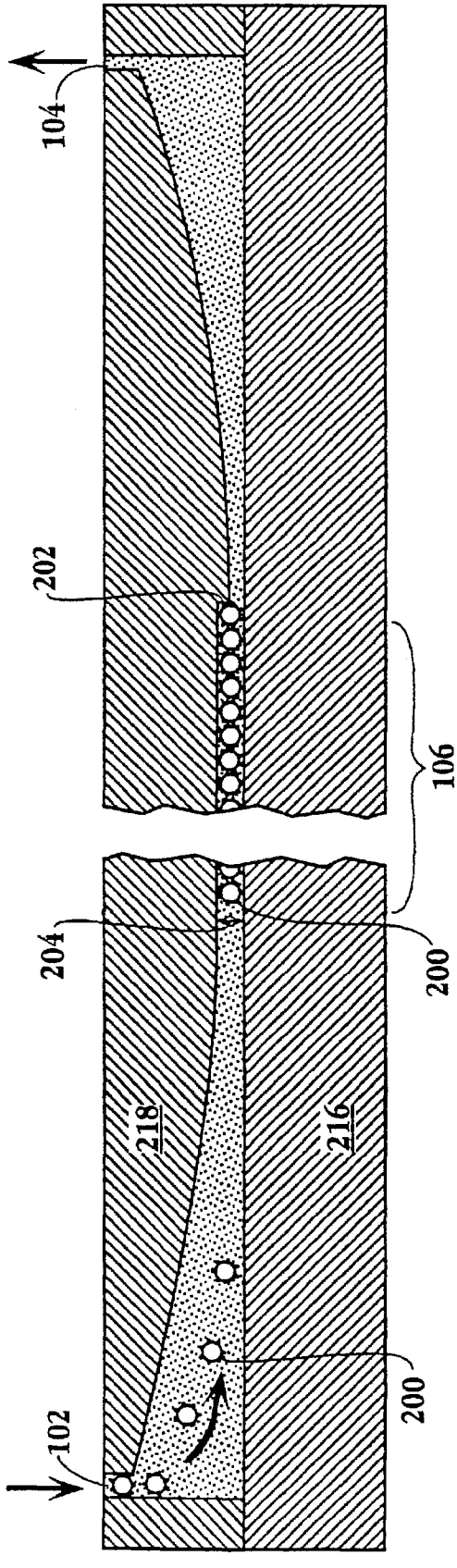


Fig. 2C

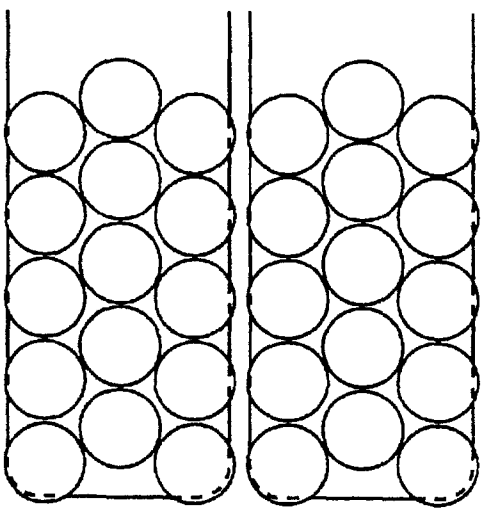


Fig. 3A

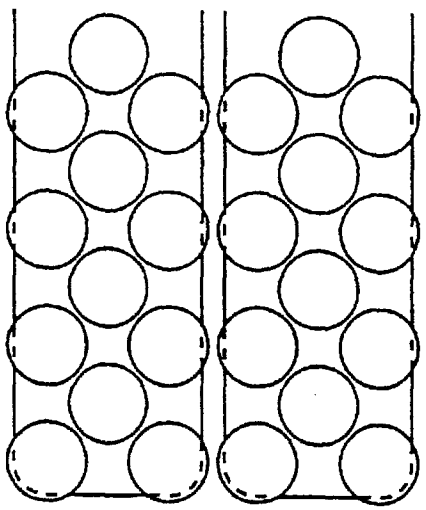


Fig. 3B



Fig. 3C



Fig. 3D

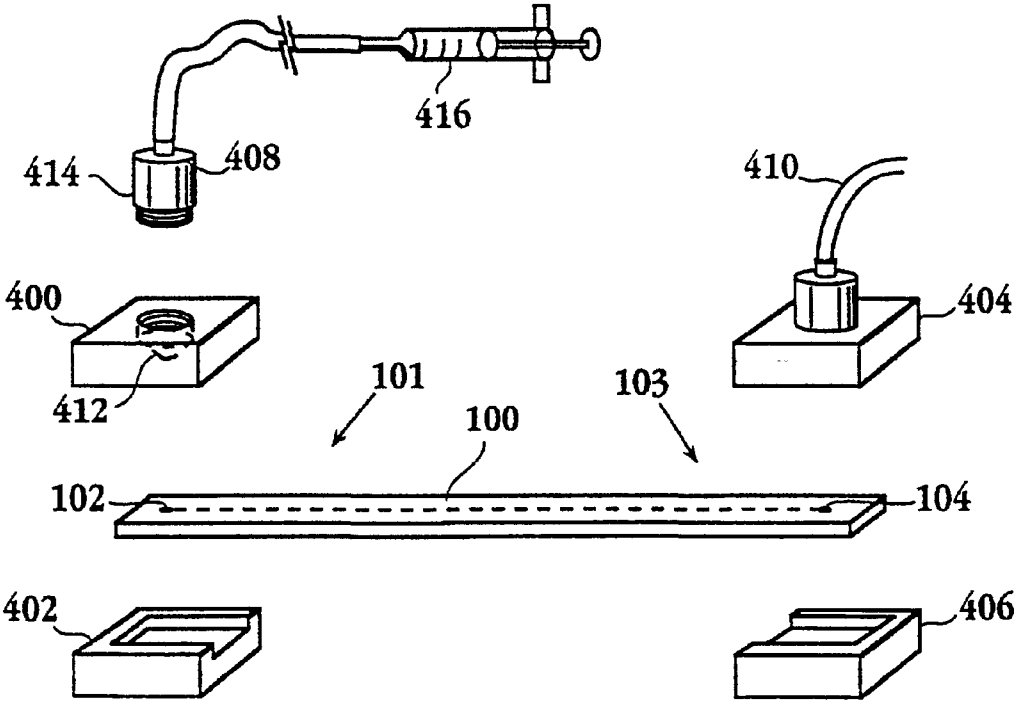


Fig. 4

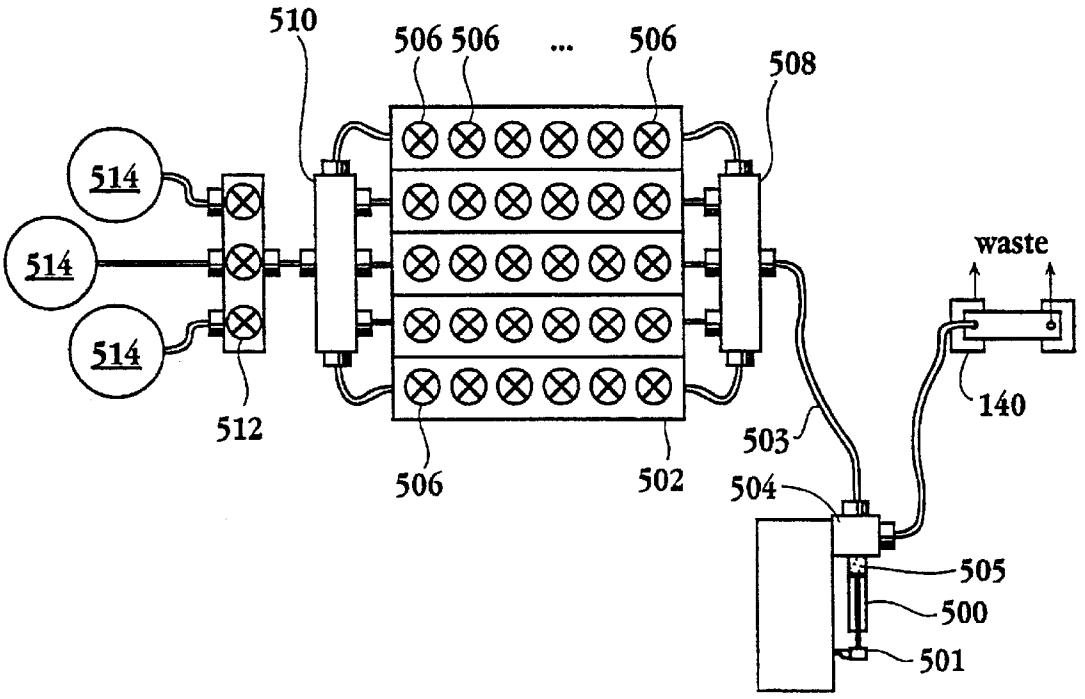

510
506 506 ... 506
508
514
514
514
512
506
502
waste
140
503
504
505
500
501

Fig. 5

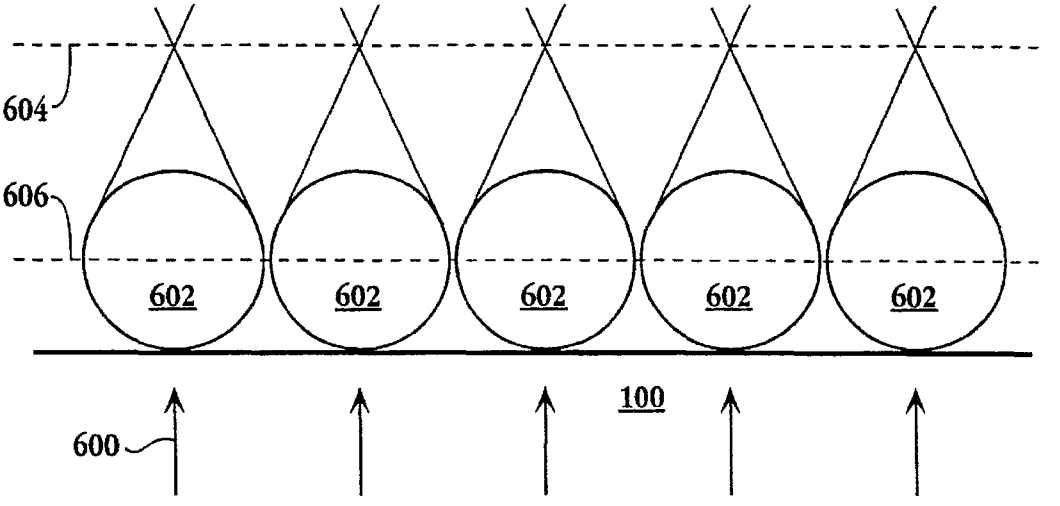

604
606
602
602
602
602
602
100
600

Fig. 6A

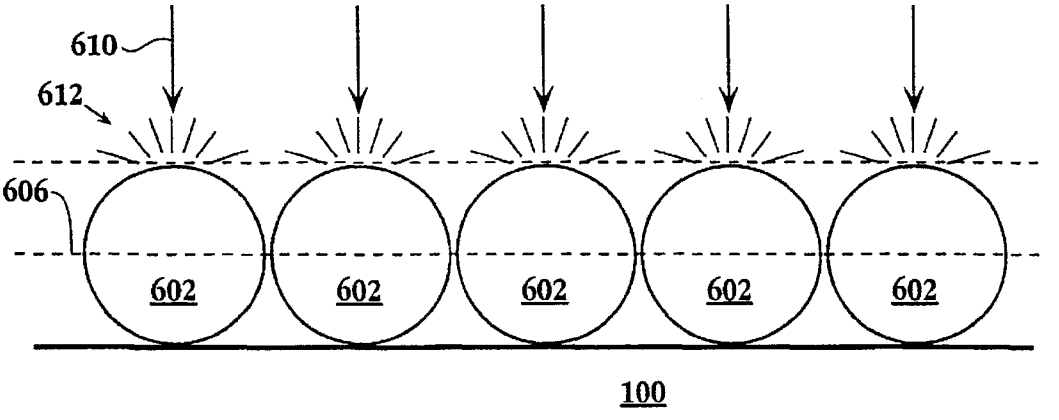

610
612
606
602
602
602
602
602
100

Fig. 6B

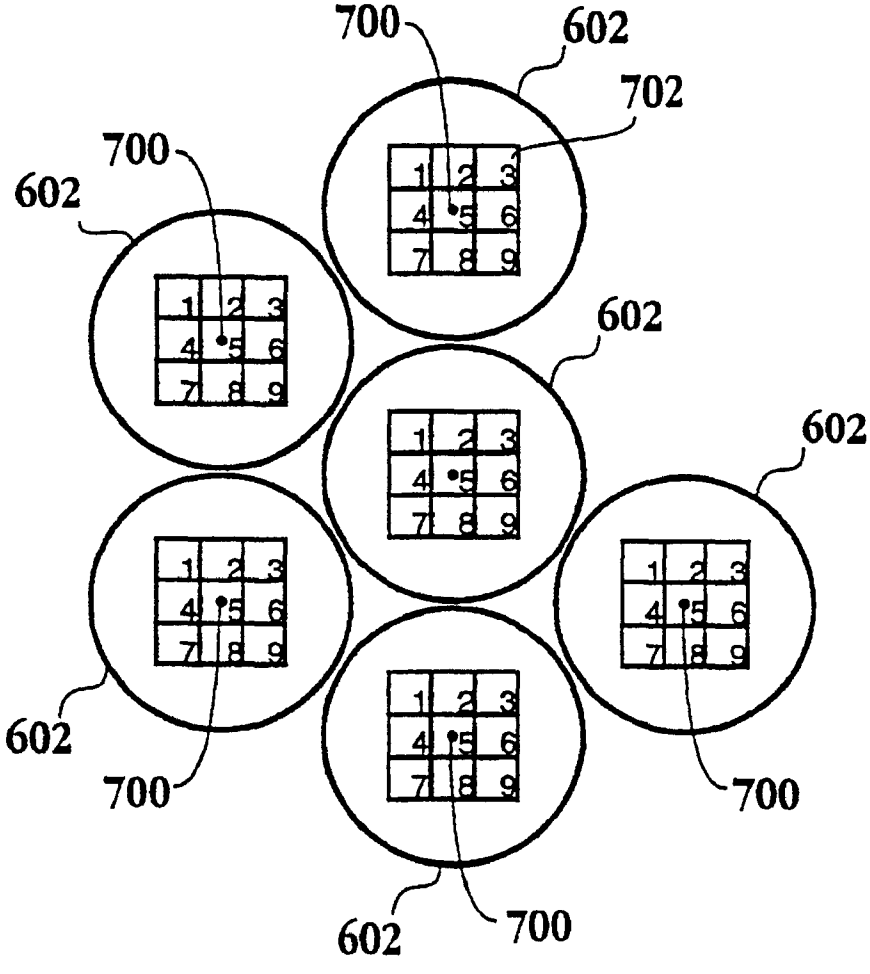


Fig. 7

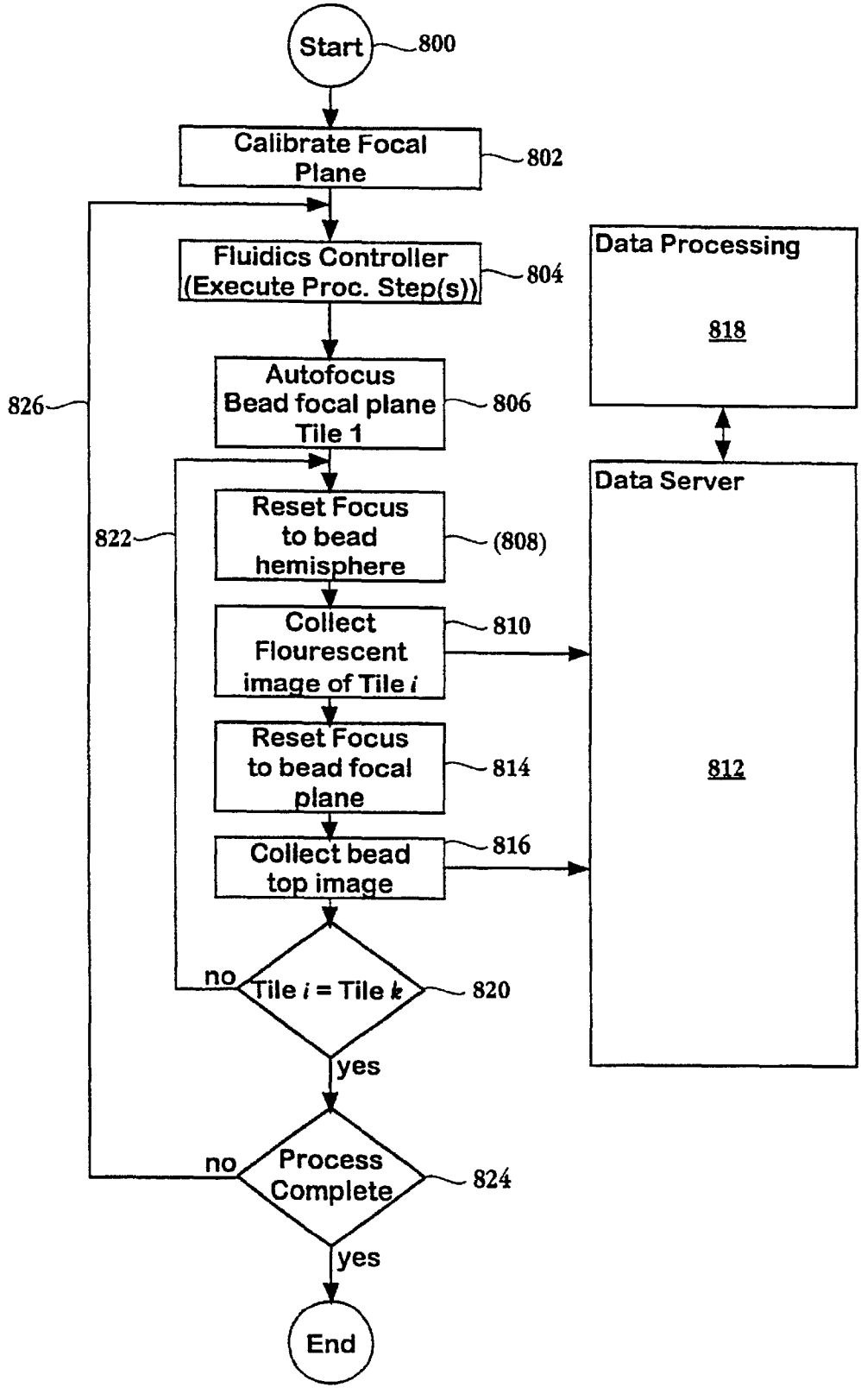

Start
800
Calibrate Focal Plane
802
Fluidics Controller (Execute Proc. Step(s))
804
Autofocus Bead focal plane Tile 1
806
826
Reset Focus to bead hemisphere
(808)
822
Collect Flourescent image of Tile i
810
Reset Focus to bead focal plane
814
Collect bead top image
816
Tile i = Tile k
820
no
yes
Process Complete
824
no
yes
End
Data Processing
818
Data Server
812

Fig. 8

SYSTEM AND APPARATUS FOR SEQUENTIAL PROCESSING OF ANALYTES

This application is a divisional of U.S. patent application Ser. No. 09/424,028, filed Nov. 16, 1999, now U.S. Pat. No. 6,406,848, which is a 371 of PCT/US98/11224, filed May 22, 1998, which is a con. of U.S. patent application Serial No. 08/862,610, filed May 23, 1997, now abandoned, all of which are incorporated in their entirety herein by reference.

FIELD OF THE INVENTION

The invention relates generally to systems and apparatus for carrying out large scale parallel reactions on solid phase supports, and more particularly, to systems and apparatus for monitoring and carrying out reactions on arrays of microparticles.

BACKGROUND

The desire to understand and analyze complex chemical and biological systems has led to the development of analytical techniques that employ parallelization and miniaturization of analyte processing, e.g. Graber et al, Current Opinion in Biotechnology, 9: 14–18 (1998); Fodor et al, Nature, 364: 555–556 (1993); Meier-Ewert et al, Nature, 361: 375–376 (1993); Taylor et al, Nucleic Acids Research, 25: 3164–3168 (1997); Garner et al, BioTechniques, 14: 112–115 (1993); Lam et al, Nature, 354: 82–84 (1991); Ohlmeyer et al, Proc. Natl. Acad. Sci., 90: 10922–10926 (1993); DeRisi et al, Science, 278: 680–686 (1997); Wodicka et al, Nature Biotechnology, 15: 1359–1367 (1997); and the like.

Many of these techniques employ microparticles for synthesizing analytes or for capturing analytes for subsequent analysis, e.g. Lam et al (cited above); Benkovic et al, International patent application PCT/US95/03355; Gavin et al, International patent application PCT/EP97/02039; Brenner et al, International patent application PCT/US96/09513, and the like. Even though the properties of different types of microparticles can vary widely, microparticles generally facilitate the construction and manipulation of large repertoires of analytes with minimal reagent and/or sample consumption. However, handling and manipulating large numbers of microparticles, e.g. tens to hundreds of thousands, for carrying out specific chemical and/or biochemical analyses gives rise to many difficulties, including whether sufficient signal is generated on individual microparticles for detection, how to track individual microparticles through multiple steps of a process, mechanical strength of microparticles under pressure or flow conditions, the ability to uniformly deliver reagents to microparticles for carrying out steps of an analytical process, whether clumping or other inappropriate interaction of microparticles and/or reagents occurs, the degree to which analytes and/or processing reagents adsorb onto vessel walls, whether protein reagents or analytes denature causing a disruption of reagent distribution and access, whether adjacent microparticles will interact, e.g. to degrade or obscure a signal or to inhibit reagent access, and the like.

In view of these difficulties, it would be desirable to provide a system and apparatus for handling and processing multiple solid phase supports, such as populations of microparticles. It would be especially desirable if such system and apparatus permitted the tracking and analysis of multiple analytes anchored to separate microparticles through a sequence of several processing and/or analysis steps.

SUMMARY OF THE INVENTION

Accordingly, objects of our invention include, but are not limited to, providing a system and apparatus for sequentially

delivering reagents to a population of analytes anchored to separate microparticles; providing an apparatus for simultaneously monitoring the interactions of processing reagents and analytes on the surfaces of microparticles disposed in a planar array; providing an apparatus for detecting optical signals generated by, or as the result of, interactions of processing reagents and analytes on the surfaces of microparticles disposed in a planar array; providing an apparatus for detecting pluralities of optical signals, each such plurality being generated at the surface of the same microparticle as a result of interactions between processing reagents and an analyte anchored to the surface of such microparticle; providing an apparatus for simultaneously tracking the positions of individual microparticles in a population of microparticles disposed in a flow chamber as a closely packed planar array; and providing a system and apparatus for simultaneously analyzing the nucleotide sequences of a population of polynucleotides anchored to microparticles disposed in a planar array in a flow chamber.

Our invention achieves these and other objects with an apparatus comprising a flow chamber for disposing a population of microparticles in a planar array; fluidic means for sequentially delivering processing reagents from one or more reagent reservoirs to the flow chamber; and detection means for detecting a sequence of optical signals from each of the microparticles of the population. Preferably, the sequences of optical signals are generated as a result of a multi-step analytical process, such as nucleic acid sequence analysis.

In one aspect, the invention provides a system for simultaneously monitoring a population of analytes which includes the apparatus of the invention, microparticles carrying the analytes, and software means for processing images of, and/or optical signals generated by, the microparticles when disposed in a planar array. Preferably, the flow chamber includes constraining means for restricting the movement of microparticles during cycles of reagent delivery.

In another aspect, the invention includes a system for simultaneously analyzing the nucleotide sequences of a population of polynucleotides. Copies of each kind of polynucleotide in the population are sorted onto and anchored to one or more microparticles so that a population of loaded microparticles is formed. Loaded microparticles are disposed in a planar array in a flow chamber through which processing reagents are sequentially delivered to the loaded microparticles from one or more reagent reservoirs by a fluidic means. Optical signals generated by, or produced as a result of, the interaction of processing reagents and polynucleotides on the microparticles are imaged by a detection means. Preferably, when analysis includes determining the nucleotide sequence of a portion of each polynucleotide on the different microparticles, massively parallel signature sequencing (MPSS) analysis is employed, e.g. as described in Albrecht et al, International patent application PCT/US97/09472.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1***a* is a schematic representation of a flow chamber and fluidics and detection systems for observing a planar array of microparticles loaded with analyte molecules, such as cDNA molecules for sequencing.

FIG. **1***b* is a schematic of a preferred holder for a flow chamber.

FIG. **2***a* is bilateral cut away view of a flow chamber.

FIG. **2***b* is a top view of a flow chamber.

FIG. **2***c* is an illustration of microparticles being loaded into a flow chamber.

FIGS. **3***a* through **3***d* schematically illustrate microparticle constraining means for a flow chamber.

FIG. **4** is a schematic representation of a device for loading microparticles into a flow chamber.

FIG. **5** is a schematic representation of a fluidics system for use with the invention.

FIGS. **6***a* and **6***b* schematically illustrate top-lighting and back-lighting approaches for determining microparticle centers in an array.

FIG. **7** schematically illustrates the assignment of pixels to microparticles for data processing.

FIG. **8** is a flow chart summarizing operation of the system of the invention.

DEFINITIONS

"Complement" or "tag complement" as used herein in reference to oligonucleotide tags refers to an oligonucleotide to which a oligonucleotide tag specifically hybridizes to form a perfectly matched duplex or triplex. In embodiments where specific hybridization results in a triplex, the oligonucleotide tag may be selected to be either double stranded or single stranded. Thus, where triplexes are formed, the term "complement" is meant to encompass either a double stranded complement of a single stranded oligonucleotide tag or a single stranded complement of a double stranded oligonucleotide tag.

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, anomeric forms thereof, peptide nucleic acids (PNAs), and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g. 3–4, to several tens of monomeric units, e.g. 40–60. Whenever an oligonucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, unless otherwise-noted. Usually oligonucleotides of the invention comprise the four natural nucleotides; however, they may also comprise non-natural nucleotide analogs. It is clear to those skilled in the art when oligonucleotides having natural or non-natural nucleotides may be employed, e.g. where processing by enzymes is called for, usually oligonucleotides consisting of natural nucleotides are required.

"Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one other such that every nucleotide in each strand undergoes Watson-Crick basepairing with a nucleotide in the other strand. The term also comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, and the like, that may be employed. In reference to a triplex, the term means that the triplex consists of a perfectly matched duplex and a third strand in which every nucleotide undergoes Hoogsteen or reverse Hoogsteen association with a basepair of the perfectly matched duplex. Conversely, a "mismatch" in a duplex between a tag and an oligonucleotide means that a pair or triplet of nucleotides in the duplex or triplex fails to undergo Watson-Crick and/or Hoogsteen and/or reverse Hoogsteen bonding.

As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543–584 (1990), or the like, with the only proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like.

As used herein "sequence determination" or "determining a nucleotide sequence" in reference to polynucleotides includes determination of partial as well as full sequence information of the polynucleotide. That is, the term includes sequence comparisons, fingerprinting, and like levels of information about a target polynucleotide, as well as the express identification and ordering of nucleosides, usually each nucleoside, in a target polynucleotide. The term also includes the determination of the identification, ordering, and locations of one, two, or three of the four types of nucleotides within a target polynucleotide. For example, in some embodiments sequence determination may be effected by identifying the ordering and locations of a single type of nucleotide, e.g. cytosines, within the target polynucleotide "CATCGC . . . " so that its sequence is represented as a binary code, e.g. "100101 . . . " for "C-(not C)-(not C)-C-(not C)-C . . . " and the like.

As used herein, the term "complexity" in reference to a population of polynucleotides means the number of different species of molecule present in the population.

DETAILED DESCRIPTION OF THE INVENTION

The system and apparatus of the invention is particularly applicable to the analysis of molecules that can be anchored in populations of duplicate copies to particulate solid phase supports. That is, in accordance with the invention, each analyte of a population is present on at least one microparticle in a quantity sufficient for the type of analysis being performed. For example, if combinatorially synthesized peptides on the microparticles are screened against a soluble receptor protein for detecting those that form stable complexes, the number of peptides available for binding on the surface of the microparticles must be large enough to generate a detectable signal when a binding event occurs. Of course, many additional factors well known in the art will present additional design constraints, such as the nature of the system for generating optical signals, the concentration of receptors, pH, salt concentration, the density and accessibility of the peptides on the microparticle surface, the solvent system employed, and the like. Analyte populations particularly relevant for use with the present apparatus include combinatorial libraries synthesized on microparticle supports, e.g as disclosed in Lam et al, Chem. Rev., 97: 411–448 (1997); or Dower et al, U.S. Pat. No. 5,708,153, and polynucleotide libraries sorted onto microparticle supports, e.g. as disclosed in Brenner (cited above).

FIG. **1***a* is a schematic representation of an embodiment of the invention for detecting fluorescent signals. Flow chamber (**100**) having inlet (**102**), outlet (**104**) and planar

cavity (**106**) holds microparticles in a planar array from which optical signals (**108**) generated by analytes and/or reactants on microparticles can be collected and imaged. Flow chamber (**100**) is operationally associated with fluidic system (**112**) and detection system (**114**), so that delivery of fluids and collection of signals is under control of computer (**116**). Preferably, optical signals are collected by microscope (**118**) and are imaged onto a solid state imaging device, such as charge-coupled device (CCD) (**120**) which is capable of generating a digital image of the physical image of the microparticle array with sufficient resolution for individual microparticles to be distinguished. For fluorescent signals, detection system (**114**) usually includes appropriate bandpass filter (**122**) for optical signal (**108**), bandpass filter (**124**) for excitation beam (**128**) generated by light source (**126**), and other standard components. As illustrated, a conventional fluorescence microscope is preferred which is configured for epiillumination. There is a great deal of guidance in the art for selecting appropriate fluorescence microscopes, e.g Wang and Taylor, editors, Fluroescence Microscopy of Living Cells in Culture, Parts A and B, Methods in Cell Biology, Vols. 29 and 30 (Academic Press, New York, 1989).

A key feature of the invention is flow chamber (**100**). Body (**130**) of flow chamber (**100**) preferably comprised inlet (**102**), outlet (**104**) and planar cavity (**106**) which are formed by standard micromachining techniques, e.g. Ekstrom et al, International patent application PCT/SE91/00327; Brown, U.S. Pat. No. 4,911,782; Harrison et al, Anal. Chem. 64: 192–1932 (1992); and the like. Transparent plate (**132**) is sealingly attached to body (**130**) to form an operational flow chamber (**100**). Body (**130**) may be constructed from any of several different materials including glass, silicon, polyethylene, polyester, teflon, other plastics, and the like. Preferably, transparent plate (**132**) is glass or quartz; and, when body (**130**) and transparent plate (**132**) are glass or silicon, transparent plate (**132**) is preferably attached to body (**130**) by anodic bonding, e.g. Pomerantz, U.S. Pat. No. 3,397,279. Key functions of the flow chamber include i) holding a population of microparticles in a substantially immobilized planar array, or monolayer, during a sequence of processing steps, ii) ensuring that processing reagents can access each microparticle during each step of a process, and iii) minimizing processing reagent usage. The degree of immobilization required may vary among different embodiments. Generally, more movement of microparticles within a planar array increases the computational and measurement burden of tracking positions of microparticles by image processing software. Design trade-offs therefore exist between the use of image processing software and the use of physical and/or chemical means for constraining microparticle movement. Preferably, physical and/or chemical means are employed to constrain microparticle movement within the planar array of microparticles in flow chamber (**100**). Such means are referred to herein as "movement constraining means." Most preferably, physical, or mechanical, movement constraining means are employed.

Preferably, microparticles are disposed in flow chamber (**100**) in a closely packed planar array. As used herein, "closely packed" in reference to a planar array means either that the number of microparticles per unit area of a planar array is at least eighty percent of the number of microparticles in a hexagonal array of equal area, or that the average distance between centers of adjacent microparticles is less than two microparticle diameters. As used herein, a "hexagonal" array of microparticles means a planar array of microparticles in which every microparticle in the array contacts at least six other adjacent microparticles, as shown in FIG. **3***a*.

Additions features of flow chamber (**100**) of a preferred embodiment are illustrated in FIGS. **2***a* through **2***c*. FIG. **2***a* is a cross sectional view along a longitudinal plane that bisects flow chamber (**100**). The same view, in a more abstracted rendition, is shown in FIG. **2***c*. In both Figures, inlet (**102**) fluidly communicates with planar cavity (**106**) and outlet (**104**). Microparticles (**200**) carrying analytes enter inlet (**102**) and are carried by a suspending buffer to planar cavity (**106**) where they become packed against dam (**202**) which prevents the microparticles from exiting the flow chamber through outlet (**104**). Structurally, dam (**202**) may be formed by a sudden reduction of the vertical dimension of planar cavity (**106**). Preferably, vertical dimension (**204**) of planar cavity (**106**) is selected so that microparticles (**200**) are constrained to a plane, i.e. a monolayer, when they pack against dam (**202**). More preferably, vertical dimension (**204**) is selected to be between about 120 to 150 percent of the diameter of the microparticles employed. For example, when microparticles are employed that have diameters of 5 μm, vertical dimension (**204**) may be 7 μm. Magnetic microparticles may be constrained to a plane and constrained from movement by applying a magnetic field so that the microparticles are attracted to the ceiling or to the floor of planar cavity (**106**). Width (**206**) of planar cavity (**106**) is not a critical dimension; however, for convenience and efficiency, width (**206**) may be selected to correspond to the dimensions of the signal collection region of detection system (**114**). Such regions labeled l through k in FIG. **2***b* are referred to herein as "tiles." That is, the region of planar cavity (**106**) occupied by microparticles may be divided into non-overlapping areas, referred to as "tiles," that cover the entire occupied region. FIG. **2***b*, which is a top view of the flow chamber of FIG. **2***a*, also shows inlet (**102**), planar cavity (**106**), dam (**202**), and outlet (**104**) that lie in sequence along axis (**217**) of flow chamber (**100**).

Many movement constraining means may be selected for use with the flow chamber, either alone or in combination. Such means include loading microparticles with trace amounts of a chemically reactive species which may be activated and cross-linked; providing physical, or mechanical structures, such as ridges, within the flow chamber; providing magnetically responsive microparticles which may be immobilized by an external magnetic field; providing a second population of microparticles that are loaded into a flow chamber after the analyte-containing population, which forces the analyte-containing population against dam (**202**); and the like. Exemplary chemically reactive species for use with nucleic acid analytes are disclosed in Summerton et al, U.S. Pat. No. 4,123,610; Gamper et al, J. Mol. Biol., 197: 349–362 (1987); Hearst, Ann. Rev. Phys. Chem. 39: 291–315 (1988); Pieles et al, Nucleic Acids Research, 17: 8967–8978 (1989); and the like.

Preferably, microparticle movement is constrained by providing a flow chamber with planar cavity (**106**) containing a plurality of ridges running parallel to axis (**217**) of the flow chamber, i.e. parallel to the direction of reagent flow, so that microparticles are arranged into rows, which may be single-file, or several microparticles wide, as shown in FIGS. **3***a* and **3***b*. The particular selection may depend on several factors, including the degree of immobilization desired, constraints imposed by the fabrication technique used to construct the flow chamber, the amount of reagent access desired, the degree to which flow resistance or back-pressure can be tolerated, and the like. FIGS. **3***a* and **3***b* illustrate two possible distances between parallel ridges. In FIG. **3***a*, the distance is selected to permit maximal packing of microparticles into a hexagonal array, and in FIG. **3***b*, the

distance is selected for less efficient packing, but for increased reagent access to microparticle surfaces. FIGS. **3***c* and **3***d* are axial views of the flow chamber showing the microparticle arrangements of FIGS. **3***a* and **3***b*, respectively.

In some embodiments, such as those employing enzymatic processes, the inner surfaces of flow chamber **(100)** may be passivated, that is, treated to render such surfaces inert and/or non-adsorbing with respect to enzymes. The type of treatment depends on the sensitivity of the enzymes used in the process, and their affinity for the surfaces. Surface treatments include silanization, e.g. with commercially available reagents (Pierce, Rockford, Ill.); and/or adsorption of various blocking polymers, such as poly-a-alanine, polyglycine, polyadenylic acid, polymaleimide, polyvinylpyrrolidone, or the like, e.g. Shofffier et al, Nucleic Acids Research, 24: 375–379 (1996). Preferably, glass inner surfaces of flow chamber **(100)** are covalently coated with a neutral coating, such as allyl methacrylate, using the technique disclosed in Sandoval et al, U.S. Pat. No. 5,326,738, which is incorporated by reference.

FIG. **1***b* illustrates flow chamber **(100)** mounted between holders **(140)** and **(142)** which sealingly connect inlet **(102)** to inlet tubing **(144)** and outlet **(104)** to outlet tubing **(146)**, respectively. Preferably, holder **(140)** contains a rotary valve (not shown) operated by actuator **(148)** that shunts fluid flowing through inlet tubing **(144)** to inlet **(102)** or to waste line **(150)**. Such a valve minimizes the amount of process reagent from a previous step that must be passed through flow chamber **(100)** prior to the initiation of the next process step. That is, such a rotary valve permits reagent in inlet tubing **(144)** to be shunted to waste and replaced by processing reagent required for the next step in the process being executed. Preferably, for use in DNA analysis, peltier block **(152)** is employed to control temperature in flow chamber **(100)** and the entire assembly including flow chamber **(100)** and peltier block **(152)** is mounted on xyz-stage **(154)** which is under control of computer **(116)**.

Preferably, microparticles are loaded into flow chamber **(100)** prior to attachment of holders **(140)** and **(142)** and the initiation of processing steps. FIG. **4** illustrates a microparticle loader for loading microparticles into flow chamber **(100)**. Flow chamber **(100)** is mounted between holders **(400)**, **(402)**, **(404)**, and **(406)**. Holders **(400)** and **(402)** sealingly clamp onto the inlet end **(101)** of flow chamber **(100)** and holders **(404)** and **(406)** sealingly clamp onto the outlet end **(103)** of flow chamber **(100)** so that inlet tubing **(408)** is in fluid communication with outlet tubing **(410)** when the microparticle loader is assembled. Inlet tubing **(408)** is connected to syringe **(416)** which is used to drive fluid through flow chamber **(100)**. Holder **(400)** is constructed to have conical passage **(412)** which narrows to match the diameter of inlet **(102)** of flow chamber **(100)**. After assembly of holders **(400)**, **(402)**, **(404)**, and **(406)** a suspension of microparticles is placed in the conical passage after which fitting **(414)** is sealingly connected to holder **(400)**. Fluid pressure and flow generated by syringe **(416)** then drives the microparticles into planar cavity **(106)** and against dam **(202)**. In a preferred embodiment which employs 5 μm diameter GMA microparticles carrying DNA, approximately 500 thousand microparticles are loaded into flow chamber **(100)** by placing 5 μL of a 100 thousand microparticle/μL solution (TE buffer, pH 8.0, Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989)) in conical passage **(412)**, attaching fitting **(414)**, and using syringe **(416)** to drive the microparticles through inlet **(102)** and into planar cavity **(106)**. After loading, holders **(400)**, **(402)**, **(404)**, and **(406)** are removed from flow chamber **(100)**, which is then mounted on the apparatus as shown in FIG. **1***b*.

Preferably, process reagents are delivered to flow chamber **(100)** by the fluidic system illustrated in FIG. **5** which has the capacity to handle many different reagents for complex analytical processes. In the illustrated embodiment, which is used in connection with DNA sequencing, the fluidics system may accommodate up to 38 reagents, including wash buffers, rinses, enzymes, hybridization probes, adaptors, and the like. Preferably, the function of the fluidics system is the sequential metering of selected processing reagents to flow chamber **(100)**. Inlet **(102)** of flow chamber **(100)** is sealingly connected to holder **(140)** which contains rotary valve (actuator shown as **148)** (not shown in FIG. **5**). The function of the rotary valve is described above. A variety of means may be employed for moving processing reagents from reservoirs, through tubing, and into flow chamber **(100)**, including gravity feed, pressure feed, and pumps, e.g. peristaltic, syringe, and the like. Preferably, common syringe pump **(500)** is employed for removing predetermined amounts processing reagents from reservoirs and for forcing such reagents through flow chamber **(100)** at a predetermined flow rate. Under control of computer **(116)**, pump **(500)** in operational association with valve block **(502)** and rotary valve **(504)** removes a predetermined amount of processing reagent from a selected reservoir by siphoning reagent out of the reservoir on the out-stroke of plunger **(501)** of pump **(500)**. On the in-stroke of plunger **(501)**, rotary valve **(504)** directs processing reagent from tubing **(503)** to reservoir **(505)** of pump **(500)**. On the out-stroke of plunger **(501)**, state of rotary valve **(504)** is changed to direct processing reagent from reservoir **(505)** to inlet tubing **(144)**. Tubing **(503)** connects rotary valve **(504)** with manifold **(508)** which, in turn, is connected to a plurality (five shown) of banks of zero dead volume valves **(506)**. Zero dead volume valves **(506)** connect individual reservoirs holding processing reagents to a common passageway (not shown in FIG. **5**) that runs through each of the banks of valves connecting to manifold **(508)**.

A preferred zero dead volume valve is described in U.S. Pat. Nos. 4,558,845 and 4,703,913, which are incorporated by reference. Process reagents from reservoirs **(514)** are distributed to the banks of dead volume valves by way of manifold **(510)**. Alternative valve blocks for controlling delivery of process reagents to flow chamber **(100)** include the valve matrix disclosed in U.S. Pat. No. 5,203,368.

An important feature of detection means **(114)** of the invention is the ability to keep track of individual microparticles through multiple process steps and/or cycles. In connection with such tracking, detection means **(114)** periodically records optical characteristics of individual microparticles that provide a close approximation microparticle centers. Preferably, when transillumination, or "back lighting" of flow chamber **(100)** is possible, the optical characteristic is the focused back light from the microparticles. That is, in reference to FIG. **6***a*, back light **(600)** passes vertically through flow chamber **(100)** where it is focused by microparticles **(602)** onto focal plane **(604)**. The image of focal plane **(604)** in this configuration appears as a field of bright points, where each point is located at the approximate center of its corresponding microparticle. In an epiillumination system, light from above flow chamber **(100)**, i.e. "top light **(610)**," is directed from a vertical direction onto microparticles **(602)** where it scatters from the top surface of the microparticles. In this configuration, the optical characteristic is the scatter center of a microparticle. Thus, an

image is collected from the plane containing scatter centers (**612**) resulting from such top lighting. As with focused back lighting, the image of the scatter centers provides a convenient way to readily determine the approximate centers of the microparticles.

In the preferred image processing approach, once microparticle centers (**700**) are determined, pixels (**702**) are assigned for determining characteristics, e.g. intensity, of an optical signal generated at each microparticle (**602**). The size of microparticle (**602**) and pixel area determine how many pixels are assigned to each microparticle. In making such an assignment, important factors include the degree to which the calculated center of a microparticle (as described above) is likely to deviate from the geometric center, the extent to which optical signal collected from the edge of an image contains spurious information (e.g. signal from an overlapping or adjacent microparticle), the uniformity of microparticle diameter and shape, and the like. In the preferred apparatus of the invention, 5 μm diameter microparticles are employed and the pixel dimensions of the CCD detector are about 0.9 μm×0.9 μm. Thus, nine pixels fit easily within the interior of a microparticle image with a margin of at least about 1 μm between any pixel and the edge of the microparticle image. In the preferred embodiment, an initial pixel is assigned which encloses the computed center of a microparticle, e.g. pixel "5" in FIG. **7**. Thereafter, additional pixels are assigned, usually the immediately adjacent pixels. Preferably, the value of the optical signal generated by a process at the surface of a microparticle is the average value of the optical signals collected by pixels assigned to that microparticle.

The general operation of the system of the preferred embodiment is summarized by the flow chart of FIG. **8**. At the start (**800**) of an analysis, microparticles with anchored analytes have been loaded into flow chamber (**100**) which has been operationally mounted in holders **140** and **142**. The initial operation is the calibration of the microparticle focal plane (**802**). That is, the vertical, or "z", position of the xyz-stage is determined which optimizes the focus of either the scatter centers of the microparticles, i.e. the microparticle tops for top-lighting, or the focus points of the microparticles for back-lighting. The optimization is carried out by a conventional autofocusing algorithm which provides an image contrast function constructed from a predetermined sample of regions within a collected image. For example, the contrast function may be evaluated iteratively for sequence of z-positions so that the differences of successive values of the contrast function can be determined. These are tested until a difference is found below a predetermined threshold, which is taken as the maximum of the contrast function. Focal plane location is taken as the z position which maximizes the image contrast function. Such calibration is carried out for each tile, if more than one tile is employed, so that a correction table is constructed of changes in stage setting values with respect to the settings of the first tile that are required to bring the system into focus upon translation to subsequent tiles. These values are stored by computer (**116**).

After calibration, process steps are initiated (**804**) by way of a fluidics controller operationally associated with computer (**116**). After process steps (**804**) are completed, stage settings are adjusted to place the first tile into focus using the autofocus algorithm (**806**), which places the focal plane of the microscope objective approximately at the tops of the microparticles. Stage settings are then adjusted (**808**) to bring the focal plane of the microscope objective to the approximate centers of the microparticles, as illustrated (**606**) in FIGS. **6***a* and **6***b*. The amount of stage movement in this re-focusing depends on the diameter of the microparticles being used. After appropriate selection of filters (**124**) and (**122**), a fluorescent image of the first tile is collected (**810**) and transferred to data server (**812**). Fluorescent images are collected on the plane of the microparticle centers because of imperfections in the planar array. That is, microparticles in planar cavity (**106**) do not lie in a perfect planar array for a variety of reasons. For example, some microparticles are elevated above others as a result of packing into the flow chamber; there is some variability in the size and shape of the microparticles; and, the floor of planar cavity (**106**) may be uneven. After the fluorescent image is collected, the focal plane of the microscope objective is returned (**814**) to the microparticle focal plane, where another image is collected (**816**) for the purpose of computing microparticle centers as described above. The image of microparticle centers is transferred to data server (**812**) where data processor (**818**) assigns pixels of the fluorescent image to each microparticle center, as described above. After the image of microparticle centers is collected (**816**), the stage is moved so that an image of the next tile can be collected (**822**). If there are no further tiles of microparticles (**820**), then the next steps and/or cycles of the process are executed (**826**). If there are no further process steps (**824**), then the process is complete and the apparatus is placed in a holding mode.

Optical signals collected in the course of analysis may be generated by a variety of mechanisms, including absorption and fluorescence, chemiluminescence, electrochemiluminescence, or bioluminescence emission. Extensive guidance is available for selecting appropriate optical signaling means, e.g. Kessler, editor, Nonradioactive Labeling and Detection of Biomolecules (Springer-Verlag, Berlin); Keller and Manak, DNA Probes, Second Edition (Stockton Press, New York, 1993); and the like. Preferably, optical signals generated in processing steps are fluorescence emissions.

Microparticles

An important feature of the system of the invention is the use of microparticles for carrying analytes. A variety of microparticles may be employed depending on particular applications. Generally, microparticles must consist of a material compatible with the reagents and chemistry of the process steps being carried out and microparticle must be substantially mechanically rigid so that they retain their shape and size during process steps. Preferably, as used herein, the term "substantially mechanically rigid" means that microparticles neither swell nor contract by more than ten percent (as measure by diameter) in any process solvent or reagent. Preferably, microparticles are microspheres of uniform size, i.e. microparticles are monodisperse. More preferably, the diameters of spherical microparticles have a coefficient of variation less than five percent, and most preferably, less than two percent. Microparticle diameters are in the range of from 0.1 μm to 100 μm. Preferably, microparticle diameters range from 1 μm to 20 μm. Most preferably, microparticle diameters are in the range of 1 to 5 μm. Suitable microparticle materials include inorganic support materials such as glass, e.g. controlled-pore glass, Balltoni beads; silica, zirconia, and the like, e.g. Weetall, Methods in Enzymology, 44: 134–148 (1976); and organic support materials such as highly cross-linked polystyrene, polyacrylate, polymethylmethacrylate, glycidylmethacrylate (GMA), Dynabeads (Dynal, Oslo, Norway), and the like, Rembaum et al, U.S. Pat. No. 4,046,720; Hodge and

Sherrington, editors, pages 435–456, Polymer-supported Reactions in Organic Synthesis (Wiley & Sons, New York, 1980); Andrus et al, U.S. Pat. No. 5,047,524; and the like.

Attaching Identical Copies of Polynucleotides to Microparticles by Solid Phase Cloning

In a preferred embodiment of the invention, identical copies of polynucleotides from a population are anchored to separate microparticles by solid phase cloning, i.e. the use of oligonucleotide tags for sorting polynucleotides onto microparticles such that only the same kind of polynucleotide will be attached to the same microparticle, e.g. Brenner, U.S. Pat. No. 5,604,097, which is incorporated by reference. This condition is accomplished by taking a sample of the full ensemble of tag-polynucleotide conjugates. (It is acceptable that identical polynucleotides have different tags, as it merely results in the same polynucleotide being operated on or analyzed twice in two different locations.) Such sampling can be carried out either overtly—for example, by taking a small volume from a larger mixture—after the tags have been attached to the polynucleotides, it can be carried out inherently as a secondary effect of the techniques used to process the polynucleotides and tags, or sampling can be carried out both overtly and as an inherent part of processing steps.

Oligonucleotide tags for use with the invention are members of a minimally cross-hybridizing set of oligonucleotides. The sequences of oligonucleotides of such a set differ from the sequences of every other member of the same set by at least two nucleotides. Thus, each member of such a set cannot form a duplex (or triplex) with the complement of any other member with less than two mismatches. Complements of oligonucleotide tags of the invention, referred to herein as "tag complements," may comprise natural nucleotides or non-natural nucleotide analogs. Tag complements are attached to microparticles.

Minimally cross-hybridizing sets of oligonucleotide tags and tag complements may be synthesized either combinatorially or individually depending on the size of the set desired and the degree to which cross-hybridization is sought to be minimized (or stated another way, the degree to which specificity is sought to be enhanced). For example, a minimally cross-hybridizing set may consist of a set of individually synthesized 10-mer sequences that differ from each other by at least 4 nucleotides, such set having a maximum size of 332 (when composed of 3 kinds of nucleotides and counted using a computer program such as disclosed in Appendix Ic of International patent application PCT/US96/09513). Alternatively, a minimally cross-hybridizing set of oligonucleotide tags may also be assembled combinatorially from subunits which themselves are selected from a minimally cross-hybridizing set. For example, a set of minimally cross-hybridizing 12-mers differing from one another by at least three nucleotides may be synthesized by assembling 3 subunits selected from a set of minimally cross-hybridizing 4-mers that each differ from one another by three nucleotides. Such an embodiment gives a maximally sized set of 9^3, or 729, 12-mers, "9" is number of oligonucleotides generated by the computer program of Appendix Ia of International patent application PCT/US96/09513, which assumes, as with the 10-mers, that only 3 of the 4 different types of nucleotides are used. The set is described as "maximal" because the computer programs disclosed in International patent application PCT/US96/09513 provide the largest set for a given input (e.g. length, composition, difference in number of nucleotides between members). Additional minimally cross-hybridizing sets may be formed from subsets of such calculated sets.

When synthesized combinatorially, an oligonucleotide tag of the invention preferably consists of a plurality of subunits, each subunit consisting of an oligonucleotide of 3 to 9 nucleotides in length wherein each subunit is selected from the same minimally cross-hybridizing set. In such embodiments, the number of oligonucleotide tags available depends on the number of subunits per tag and on the length of the subunits.

As used herein in reference to oligonucleotide tags and tag complements, the term "repertoire" means the set of minimally cross-hybridizing set of oligonucleotides that make up the tags in a particular embodiment or the corresponding set of tag complements.

Preferably, in constructing a cDNA library where substantially all different cDNAs have different tags, a tag repertoire is employed whose complexity, or number of distinct tags, greatly exceeds the total number of mRNAs extracted from a cell or tissue sample. Preferably, the complexity of the tag repertoire is at least 10 times that of the polynucleotide population; and more preferably, the complexity of the tag repertoire is at least 10 times that of the polynucleotide population. Below, a protocol is disclosed for cDNA library construction using a primer mixture that contains a full repertoire of exemplary 9-word tags. Such a mixture of tag-containing primers has a complexity of 8^9, or about 1.34×10^8. As indicated by Winslow et al, Nucleic Acids Research, 19: 3251–3253 (1991), mRNA for library construction can be extracted from as few as 10–100 mammalian cells. Since a single mammalian cell contains about 5×10^5 copies of mRNA molecules of about 3.4×10^4 different kinds, by standard techniques one can isolate the mRNA from about 100 cells, or (theoretically) about 5×10^7 mRNA molecules. Comparing this number to the complexity of the primer mixture shows that without any additional steps, and even assuming that mRNAs are converted into cDNAs with perfect efficiency (1% efficiency or less is more accurate), the cDNA library construction protocol results in a population containing no more than 37% of the total number of different tags. That is, without any overt sampling step at all, the protocol inherently generates a sample that comprises 37%, or less, of the tag repertoire. The probability of obtaining a double under these conditions is about 5%, which is within the preferred range. With mRNA from 10 cells, the fraction of the tag repertoire sampled is reduced to only 3.7%, even assuming that all the processing steps take place at 100% efficiency. In fact, the efficiencies of the processing steps for constructing cDNA libraries are very low, a "rule of thumb" being that good library should contain about 10^8 cDNA clones from mRNA extracted from 10^6 mammalian cells.

Use of larger amounts of mRNA in the above protocol, or for larger amounts of polynucleotides in general, where the number of such molecules exceeds the complexity of the tag repertoire, a tag-polynucleotide conjugate mixture potentially contains every possible pairing of tags and types of mRNA or polynucleotide. In such cases, overt sampling may be implemented by removing a sample volume after a serial dilution of the starting mixture of tag-polynucleotide conjugates. The amount of dilution required depends on the amount of starting material and the efficiencies of the processing steps, which are readily estimated.

If mRNA were extracted from 10^6 cells (which would correspond to about 0.5 μg of poly(A)$^+$ RNA), and if primers were present in about 10–100 fold concentration excess—as is called for in a typical protocol, e.g. Sambrook et al, Molecular Cloning, Second Edition, page 8.61 [10 μL 1.8 kb mRNA at 1 mg/mL equals about 1.68×10^{-11} moles and 10

μL 18-mer primer at 1 mg/mL equals about 1.68×10^{-9} moles], then the total number of tag-polynucleotide conjugates in a cDNA library would simply be equal to or less than the starting number of mRNAs, or about 5×10^{11} vectors containing tag-polynucleotide conjugates—again this assumes that each step in cDNA construction—first strand synthesis, second strand synthesis, ligation into a vector—occurs with perfect efficiency, which is a very conservative estimate. The actual number is significantly less.

If a sample of n tag-polynucleotide conjugates are randomly drawn from a reaction mixture—as could be effected by taking a sample volume, the probability of drawing conjugates having the same tag is described by the Poisson distribution, $P(r)=e^{-\lambda}(\lambda)^r/r$, where r is the number of conjugates having the same tag and $\lambda=np$, where p is the probability of a given tag being selected. If $n=10^6$ and $p=1/(1.34\times10^8)$,then $\lambda=0.00746$ and $P(2)=2.7\times10^{-5}$. Thus, a sample of one million molecules gives rise to an expected number of doubles well within the preferred range. Such a sample is readily obtained as follows: Assume that the 5×10^{11} mRNAs are perfectly converted into 5×10^{11} vectors with tag-cDNA conjugates as inserts and that the 5×10^{11} vectors are in a reaction solution having a volume of 100 μl. Four 10-fold serial dilutions may be carried out by transferring 10 μl from the original solution into a vessel containing 90 μl of an appropriate buffer, such as TE. This process may be repeated for three additional dilutions to obtain a 100 μl solution containing 5×10^5 vector molecules per μl. A 2 μl aliquot from this solution yields 10^6 vectors containing tag-cDNA conjugates as inserts. This sample is then amplified by straight forward transformation of a competent host cell followed by culturing.

Of course, as mentioned above, no step in the above process proceeds with perfect efficiency. In particular, when vectors are employed to amplify a sample of tag-polynucleotide conjugates, the step of transforming a host is very inefficient. Usually, no more than 1% of the vectors are taken up by the host and replicated. Thus, for such a method of amplification, even fewer dilutions would be required to obtain a sample of 10^6 conjugates.

A repertoire of oligonucleotide tags can be conjugated to a population of polynucleotides in a number of ways, including direct enzymatic ligation, amplification, e.g. via PCR, using primers containing the tag sequences, and the like. The initial ligating step produces a very large population of tag-polynucleotide conjugates such that a single tag is generally attached to many different polynucleotides. However, as noted above, by taking a sufficiently small sample of the conjugates, the probability of obtaining "doubles," i.e. the same tag on two different polynucleotides, can be made negligible. Generally, the larger the sample the greater the probability of obtaining a double. Thus, a design trade-off exists between selecting a large sample of tag-polynucleotide conjugates—which, for example, ensures adequate coverage of a target polynucleotide in a shotgun sequencing operation or adequate representation of a rapidly changing mRNA pool, and selecting a small sample which ensures that a minimal number of doubles will be present. In most embodiments, the presence of doubles merely adds an additional source of noise or, in the case of sequencing, a minor complication in scanning and signal processing, as microparticles giving multiple fluorescent signals can simply be ignored.

As used herein, the term "substantially all" in reference to attaching tags to molecules, especially polynucleotides, is meant to reflect the statistical nature of the sampling procedure employed to obtain a population of tag-molecule conjugates essentially free of doubles. The meaning of substantially all in terms of actual percentages of tag-molecule conjugates depends on how the tags are being employed. Preferably, for nucleic acid sequencing, substantially all means that at least eighty percent of the polynucleotides have unique tags attached. More preferably, it means that at least ninety percent of the polynucleotides have unique tags attached. Still more preferably, it means that at least ninety-five percent of the polynucleotides have unique tags attached. And, most preferably, it means that at least ninety-nine percent of the polynucleotides have unique tags attached.

Tags can be conjugated to cDNAs of existing libraries by standard cloning methods. cDNAs are excised from their existing vector, isolated, and then ligated into a vector containing a repertoire of tags. Preferably, the tag-containing vector is linearized by cleaving with two restriction enzymes so that the excised cDNAs can be ligated in a predetermined orientation. The concentration of the linearized tag-containing vector is in substantial excess over that of the cDNA inserts so that ligation provides an inherent sampling of tags.

A general method for exposing the single stranded tag after amplification involves digesting a target polynucleotide-containing conjugate with the 5'3' exonuclease activity of T4 DNA polymerase, or a like enzyme, e.g. as described in Kuijper et al, Gene, 112: 147–155 (1992). When used in the presence of a single deoxynucleoside triphosphate, such a polymerase will cleave nucleotides from 3' recessed ends present on the non-template strand of a double stranded fragment until a complement of the single deoxynucleoside triphosphate is reached on the template strand. When such a nucleotide is reached the 5'→3' digestion effectively ceases, as the polymerase's extension activity adds nucleotides at a higher rate than the excision activity removes nucleotides. Consequently, single stranded tags constructed with three nucleotides are readily prepared for loading onto solid phase supports.

After the oligonucleotide tags are prepared for specific hybridization, e.g. by rendering them single stranded as described above, the polynucleotides are mixed with microparticles containing the complementary sequences of the tags under conditions that favor the formation of perfectly matched duplexes between the tags and their complements. There is extensive guidance in the literature for creating these conditions. Exemplary references providing such guidance include Wetmur, Critical Reviews in Biochemistry and Molecular Biology, 26: 227–259 (1991); Sambrook et al, Molecular Cloning: A Laboratory Manual, 2nd Edition (Cold Spring Harbor Laboratory, New York, 1989); and the like. Preferably, the hybridization conditions are sufficiently stringent so that only perfectly matched sequences form stable duplexes. Under such conditions the polynucleotides specifically hybridized through their tags may be ligated to the complementary sequences attached to the microparticles. Finally, the microparticles are washed to remove polynucleotides with unligated and/or mismatched tags.

Preferably, for sequencing applications, standard CPG beads of diameter in the range of 20–50 μm are loaded with about 10^5 polynucleotides, and glycidalmethacrylate (GMA) beads available from Bangs Laboratories (Carmel, Ind.) of diameter in the range of 5–10 μm are loaded with a few tens of thousand polynucleotide, e.g. 4×10^4 to 6×10^4, to a hundred thousand polynucleotides.

DNA Sequencing

Polynucleotides loaded onto microparticles may be simultaneously sequenced in the instant apparatus using a "base-by-base" DNA sequencing methodology. Such sequencing methodology permits the stepwise identification of a sequence of nucleotides in a target polynucleotide, usually one base at a time, through successive cycles of treatment and detection. Base-by-base approaches are disclosed in the following references: Cheeseman, U.S. Pat. No. 5,302,509; Tsien et al, International application WO 91/06678; Rosenthal et al, International application WO 93/21340; Canard et al, Gene, 148: 1–6 (1994); Metzker et al, Nucleic Acids Research, 22: 4259–4267 (1994); and the like. Preferably, the base-by-base approach disclosed by Brenner in U.S. Pat. No. 5,599,675 is used with the apparatus of the invention to sequence polynucleotides on a population of loaded microparticles disposed as a planar array in the flow chamber. Accordingly, Brenner, U.S. Pat. No. 5,599,675 is incorporated by reference. Preferably, the a population of loaded microparticles for sequencing includes at least ten thousand loaded microparticles; more preferably, such a population includes at least fifty thousand loaded microparticles; and still more preferably, such a population includes at least one hundred thousand loaded microparticles.

Preferably, the sequencing method of Brenner (cited above) is employed in the embodiment disclosed in Albrecht et al International patent application PCT/US97/109472 which discloses the use of encoded adaptors. An encoded adaptor is a double stranded oligonucleotide comprising a protruding strand and an oligonucleotide tag selected from a minimally cross-hybridizing set of oligonucleotides. Encoded adaptors whose protruding strands form perfectly matched duplexes with the complementary protruding strands of the target polynucleotide are ligated. After ligation, the identity and ordering of the nucleotides in the protruding strands are determined, or "decoded," by specifically hybridizing a labeled tag complement to its corresponding tag on the ligated adaptor. Encoded adaptors may be used in an adaptor-based method of DNA sequencing that includes repeated cycles of ligation, identification, and cleavage, such as the method described in Brenner (cited above). Briefly, such a method comprises the following steps: (a) ligating an encoded adaptor to an end of a polynucleotide, the encoded adaptor having a nuclease recognition site of a nuclease whose cleavage site is separate from its recognition site; (b) identifying one or more nucleotides at the end of the polynucleotide by the identity of the encoded adaptor ligated thereto; (c) cleaving the polynucleotide with a nuclease recognizing the nuclease recognition site of the encoded adaptor such that the polynucleotide is shortened by one or more nucleotides; and (d) repeating said steps (a) through (c) until said nucleotide sequence of the polynucleotide is determined. In the identification step, successive sets of tag complements are specifically hybridized to the respective tags carried by encoded adaptors ligated to the ends of the target polynucleotides, as described above. The type and sequence of nucleotides in the protruding strands of the polynucleotides are identified by the label carried by the specifically hybridized tag complement and the set from which the tag complement came.

Construction and Sorting of cDNA Library for Signature Sequencing with Encoded Adaptors

In this example, a cDNA library is constructed in which an oligonucleotide tag consisting of 8 four-nucleotide "words" is attached to each cDNA. As described above, the repertoire of oligonucleotide tags of this size is sufficiently large (about 10^8) so that if the cDNAs are synthesized from a population of about 10^6 mRNAs, then there is a high probability that each cDNA will have a unique tag for sorting. After mRNA extraction, first strand synthesis is carried out in the presence of 5-Me-dCTP (to block certain cDNA restriction sites) and a biotinylated primer mixture containing the oligonucleotide tags. After conventional second strand synthesis, the tag-cDNA conjugates are cleaved with Dpn II (which is unaffected by the 5-Me-deoxycytosines), the biotinylated portions are separated from the reaction mixture using streptavidin-coated magnetic beads, and the tag-cDNA conjugates are recovered by cleaving them from the magnetic beads via a Bsm BI site carried by the biotinylated primer. The Bsm BI-Dpn II fragment containing the tag-cDNA conjugate is then inserted into a plasmid and amplified. After isolation of the plasmids, tag-cDNA conjugates are amplified out of the plasmids by PCR in the presence of 5-Me-dCTP, using biotinylated and fluorescently labeled primers containing pre-defined restriction endonuclease sites. After affinity purification with streptavidin coated magnetic beads, the tag-cDNA conjugates are cleaved from the beads, treated with T4 DNA polymerase in the presence of dGTP to render the tags single stranded, and then combined with a repertoire of GMA beads having tag complements attached. After stringent hybridization and ligation, the GMA beads are sorted via FACS to produce an enriched population of GMA beads loaded with cDNAs. The enriched population of loaded GMA beads are immobilized in a planar array in a flow chamber where base-by-base sequence takes place using encoded adaptors, as disclosed in Albrecht et al, International patent application PCT/US97/09472.

Approximately 5 μg of poly(A^+) mRNA is extracted from DBY746 yeast cells using conventional protocols. First and second strand cDNA synthesis is carried out by combining 100–150 pmoles of the following primer (SEQ ID NO: 1):

5'-biotin-
ACTAATCGTCTCACTATTTAATTAA[W,W,W,G]$_8$CC(T)$_{18}$V-3'

with the poly(A^+) mRNA using a Stratagene (La Jolla, Calif.) cDNA Synthesis Kit in accordance with the manufacturer's protocol. This results in cDNAs whose first stand deoxycytosines are methylated at the 5-carbon position. In the above formula, "V" is G, C, or A, "[W,W,W,G]" is a four-nucleotide word selected from Table II of Brenner, International patent application PCT/US96/09513, the single underlined portion is a Bsm BI recognition site, and the double underlined portion is a Pac I recognition site. After size fractionation (GIBCO-BRL cDNA Size Fractionation Kit) using conventional protocols, the cDNAs are digested with Dpn II (New England Bioscience, Beverly, Mass.) using manufacturer's protocol and affinity purified with streptavidin-coated magnetic beads (M-280 beads, Dynal A. S., Oslo, Norway). The DNA captured by the beads is digested with Bsm BI to release the tag-cDNA conjugates for cloning into a modified pBCSK$^-$ vector (Stratagene, La Jolla, Calif.) using standard protocols. The pBCSK$^-$ vector is modified by adding a Bbs I site by inserting the following fragment (SEQ ID NO: 2) into the Kpn I/Eco RV digested vector.

CGAAGACCC
3'-CATGGCTTCTGGGGATA-5'

Bsm BI/Dpn II digested tag-cDNA conjugate is inserted in the pBCSK⁻ which is previously digested with Bbs I and Bam HI. After ligation, the vector is transfected into the manufacturer's recommended host for amplification.

After isolating the above pBCSK⁻ vector from a standard plasmid miniprep, the tag-cDNA conjugates are amplified by PCR in the presence of 5-Me-dCTP using 20-mer primers complementary to vector sequences flanking the tag-cDNA insert. The "upstream" primer, i.e. adjacent to the tag, is biotinylated and the "downstream" primer, i.e. adjacent to the cDNA, is labeled with fluorescein. After amplification, the PCR product is affinity purified then cleaved with Pac I to release fluorescently labeled tag-cDNA conjugates. The tags of the conjugates are rendered single stranded by treating them with T4 DNA polymerase in the presence of dGTP. After the reaction is quenched, the tag-cDNA conjugate is purified by phenol-chloroform extraction and combined with 5.5 mm GMA beads carrying tag complements, each tag complement having a 5' phosphate. Hybridization is conducted under stringent conditions in the presence of a thermal stable ligase so that only tags forming perfectly matched duplexes with their complements are ligated. The GMA beads are washed and the loaded beads are concentrated by FACS sorting, using the fluorescently labeled cDNAs to identify loaded GMA beads. The tag-cDNA conjugates attached to the GMA beads are digested with Dpn II to remove the fluorescent label and treated with alkaline phosphatase to prepare the cDNAs for sequencing. That is, phasphatase is used to remove the 5' phosphate from the ends of the cDNAs to prevent unwanted cDNA-cDNA ligations by way of the palindromic Dpn II site.

The following cleavage adaptor (SEQ ID NO: 3) is ligated to the Dpn II-digested and phosphatase treated cDNAs:

```
5'-pGATCAGCTGCTGCAAATTT
      pTCGACGACGTTTAAA
```

After ligation, the 3' phosphate is removed by alkaline phosphatase, the 5' strand of the cDNA is treated with T4 DNA kinase, and the nick between the cleavage adaptor and cDNA is ligated. After cleavage by Bbv I, encoded adaptors are ligated to the ends of the cDNAs and the beads are ready for loading into the flow chamber.

Ligation of the adaptors to the target polynucleotide is carried out in a mixture consisting of 5 μl beads (20 mg), 3 μL NEB 10× ligase buffer, 5 μL adaptor mix (25 nM), 2.5 μL NEB T4 DNA ligase (2000 units/μL), and 14.5 μL distilled water. The mixture is incubated at 16° C. for 30 minutes, after which the beads are washed 3 times in TE (pH 8.0).

After centrifugation and removal of TE, the 3' phosphates of the ligated adaptors are removed by treating the polynucleotide-bead mixture with calf intestinal alkaline phosphatase (CIP) (New England Biolabs, Beverly, Mass.), using the manufacturer's protocol. After removal of the 3' phosphates, the CIP may be inactivated by proteolytic digestion, e.g. using Pronase™ (available form Boeringer Mannhiem, Indianapolis, Ind.), or an equivalent protease, with the manufacturer's protocol. The polynucleotide-bead mixture is then washed, treated with a mixture of T4 polynucleotide kinase and T4 DNA ligase (New England Biolabs, Beverly, Mass.) to add a 5' phosphate at the gap between the target polynucleotide and the adaptor, and to complete the ligation of the adaptors to the target polynucleotide. The bead-polynucleotide mixture is then washed in TE, diluted to a concentration of approximately 100 thousand beads per μL, and 5 μL of the resulting solution is loaded into a flow chamber with the help of the holders of FIG. **4**.

The top strands of the following 16 sets of 64 encoded adaptors (SEQ ID NO: 4 through SEQ ID NO: 19) are each separately synthesized on an automated DNA synthesizer (model 392 Applied Biosystems, Foster City) using standard methods. The bottom strand, which is the same for all adaptors, is synthesized separately then hybridized to the respective top strands:

SEQ ID NO.	Encoded Adaptor
4	5'-pANNNTACAGCTGCATCCCttggcgctgagg pATGCACGCGTAGGG-5'
5	5'-pNANNTACAGCTGCATCCCtgggcctgtaag pATGCACGCGTAGGG-5'
6	5'-pCNNNTACAGCTGCATCCCttgacgggtctc pATGCACGCGTAGGG-5'
7	5'-pNCNNTACAGCTGCATCCCtgcccgcacagt pATGCACGCGTAGGG-5'
8	5'-pGNNNTACAGCTGCATCCCttcgcctcggac pATGCACGCGTAGGG-5'
9	5'-pNGNNTACAGCTGCATCCCtgatccgctagc pATGCACGCGTAGGG-5'
10	5'-pTNNNTACAGCTGCATCCCttccgaacccgc pATGCACGCGTAGGG-5'
11	5'-pNTNNTACAGCTGCATCCCtgaggggatag pATGCACGCGTAGGG-5'
12	5'-pNNANTACAGCTGCATCCCttcccgctacac pATGCACGCGTAGGG-5'
13	5'-pNNNATACAGCTCCATCCCtgactccccgag pATGCACGCGTAGGG-5'
14	5'-pNNCNTACAGCTGCATCCCtgtgttgcgcgg pATGCACGCGTAGGG-5'
15	5'-pNNNCTACAGCTGCATCCCtctacagcagcg pATGCACGCGTAGGG-5'
16	5'-pNNGNTACAGCTGCATCCCtgtcgcgtcgtt pATGCACGCGTAGGG-5'
17	5'-pNNNGTACAGCTGCATCCCtcggagcaacct pATGCACGCGTAGGG-5'
18	5'-pNNTNTACAGCTGCATCCCtggtgaccgtag pATGCACGCGTAGGG-5'
19	5'-pNNNTTACAGCTGCATCCCtcccctgtcgga pATGCACGCGTAGGG-5'

where N and p are as defined above, and the nucleotides indicated in lower case letters are the 12-mer oligonucleotide tags. Each tag differs from every other by 6 nucle-

otides. Equal molar quantities of each adaptor are combined in NEB #2 restriction buffer (New England Biolabs, Beverly, Mass.) to form a mixture at a concentration of 1000 pmol/μL.

Each of the 16 tag complements are separately synthesized as amino-derivatized oligonucleotides and are each labeled with a fluorescein molecule (using an NHS-ester of fluorescein, available from Molecular Probes, Eugene, Oreg.) which is attached to the 5' end of the tag complement through a polyethylene glycol linker (Clonetech Laboratories, Palo Alto, Calif.). The sequences of the tag complements are simply the 12-mer complements of the tags listed above.

A flow chamber of the design shown in FIGS. **2***a* and **2***b* is employed in association with an Olympus Optical Co., Ltd. (Tokyo, Japan) model BX60MF5 fluorescent microscope fitted with a model U-ULS75XE 75 watt Xenon arc lamp, a motorized filter wheel, a Ludl Electronic Products, Ltd. computer-controlled stage, and a Photometrics, Ltd. (Tucson, Ariz.) PXL CCD camera with a 2000×2000 pixel array. Appropriate bandpass filters **(122)** and **(124)** are employed for exciting fluorescein and transmitting fluorescent signal to CCD camera **(120)**. Microparticle positions are determined by top-lighting with broadband light from Xenon lamp **(126)** reduced by a factor of about 10^{-4} with a neutral density filter. Fluorescent images are collected with about 2 minute exposure times.

Height **(204)** of flow chamber **(201)** is selected to be 7 μm, or approximately 140% of the diameter of the GMA beads. Width **(210)** of flow chamber **(201)** is selected so as to ensure that a 3×3 array of 9 image pixels will cover approximately 40–60% of a bead's image after 10× magnification (as illustrated in FIG. **7**). Thus, in order to capture images of tiles of about 100 thousand 5 μm GMA beads, width **(210)** is selected to have a value of 1.7 mm. Length **(212)** is selected so that the flow chamber can hold from 1 to 10 tiles of about one hundred thousand 5 μm diameter beads each. The cross section **(220)** of inlet passage **(214)** matches that of the inlet tubing and gradually enlarges to match that of flow chamber **(201)** in the region of the planar cavity, i.e. the region holding the GMA beads on which analysis is performed. It is desirable to have a constant cross section through the planar cavity of flow chamber **(201)** to minimize the creation of non-uniform flow patterns, as might occur with sudden constrictions and/or expansions in cross section. Both body **(218)** and cover **(216)** of flow chamber **(201)** are glass. and the planar cavity and channels of body **(218)** are formed by standard chemical etching techniques. Cross section **(222)** of outlet passage **(224)** is selected to match the cross section of flow chamber **(201)** at dam **(202)**.

The fluidics system of FIG. **5***a* which includes all valves, syringe pump **(500)**, and Peltier block **(152)**, is controlled by code written in LabVIEW 5.0 (National Instruments, Austin, Tex.) and run on a Compact Deskpro Pentium-based microprocessor, which is connected to the various components of the fluidics system by standard I/O circuit boards. Detection system **(114)** and overall control of the instrument is effected through a Sun Microsystems (Mountain View, Calif.) Sparcstation 5.

Three cycles of ligation, identification, and cleavage are carried out in flow chamber **(201)** to give the sequences of 12 nucleotides at the termini of each of approximately 500,000 cDNAs. That is, five tiles of GMA beads are analyzed in the following series of process steps:

1. Calibrate focal plane of GMA beads.
2. Hybridize decoder.
3. Autofocus on tile **1**.
4. Set focus to bead centers.
5. Collect fluorescent image.
6. Set focus to bead focal plane (scatter centers).
7. Collect image.
8. Repeat steps 4–7 for remaining tiles.
9. Wash.
10. Repeat steps 2–9 for remaining decoders.
11. Cleave encoded adaptor.
12. Wash.
13. Ligate top strand of next encoded adaptor.
14. Wash.
15. Repeat steps 13–14.
16. Kinase bottom strand of encoded adaptor.
17. Wash.
18. Ligate bottom strand of encoded adaptor.
19. Wash.
20. Repeat steps 2–9.
21. Repeat steps 11–19 for next encoded adaptor.

In steps 2–9, nucleotides of the cDNAs are identified by hybridizing tag complements to the encoded adaptors. Specifically hybridized tag complements are detected by exciting their fluorescent labels with illumination beam **(110)** from Xenon arc lamp **(126)**. In step 13, encoded adaptors and T4 DNA ligase (Promega, Madison, Wis.) at about 0.75 units per μL are passed through the flow chamber at a flow rate of about 1–2 μL per minute for about 20–30 minutes at 16° C., after which wash of step 14 is executed by flowing, in succession, a solution of Pronase™ (Boehringer Mannheim, Indianapolis, Ind.), a salt wash solution, and an ethanol wash solution through the flow chamber, all with the same flow rate of 1–2 μL per minute and for durations of 15, 10, and 10 minutes, respectively. The salt wash solution is 150 mM NaCl and 10 mM Tris-HCl (pH 8.5), and the ethanol wash solution is 3:1 (v/v) solution of the salt wash solution and ethanol. The ligation and wash steps 13 and 14 are repeated once, after which the adaptors and the cDNAs are prepared for second strand ligation by passing T4 DNA kinase (New England Bioscience, Beverly, Mass.) at 7 units per μL through the flow chamber at 37° C. with a flow rate of 1–2 μL per minute for 15–20 minutes. Ligation of the second strand is carried out by flowing T4 DNA ligase (0.75 units per mL, Promega) through the flow chamber for 20–30 minutes at a rate of 1–2 μL per minute, followed by Pronase™ treatment and washing as described above. Tag complements at 25 nM concentration are passed through the flow chamber at a flow rate of 1–2 μL per minute for 10 minutes at 20° C., after which the fluorescent labels carried by the tag complements are illuminated and fluorescence is collected. The tag complements are melted from the encoded adaptors by passing NEB #2 restriction buffer with 3 mM $MgCl_2$ through the flow chamber at a flow rate of 1–2 μL per minute at 55° C. for 10 minutes. Encoded adaptors are cleaved from the cDNAs by passing Bbv I (New England Biosciences, Beverly, Mass.) at 1 unit/μL at a flow rate of 1–2 μL per minute for 20 minutes at 37° C., followed by Pronase™ treatment and washing, as described above.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 19

<210> SEQ ID NO 1
<211> LENGTH: 78
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer
<221> NAME/KEY: misc_feature
<222> LOCATION: (26)...(57)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 1

actaatcgtc tcactattta attaannnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnggt 60

ttttttttt tttttttv 78

<210> SEQ ID NO 2
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic fragment

<400> SEQUENCE: 2

ataggggtct tcggtac 17

<210> SEQ ID NO 3
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic cleavage adaptor

<400> SEQUENCE: 3

gatcagctgc tgcaaattt 19

<210> SEQ ID NO 4
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 4

annntacagc tgcatccctt ggcgctgagg 30

<210> SEQ ID NO 5
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 5

nanntacagc tgcatccctg ggcctgtaag 30

<210> SEQ ID NO 6
<211> LENGTH: 30
<212> TYPE: DNA

-continued

<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 6

cnnntacagc tgcatccctt gacgggtctc 30

<210> SEQ ID NO 7
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 7

ncnntacagc tgcatccctg cccgcacagt 30

<210> SEQ ID NO 8
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 8

gnnntacagc tgcatccctt cgcctcggac 30

<210> SEQ ID NO 9
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 9

ngnntacagc tgcatccctg atccgctagc 30

<210> SEQ ID NO 10
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 10

tnnntacagc tgcatccctt ccgaacccgc 30

<210> SEQ ID NO 11
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature

-continued

<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 11

ntnntacagc tgcatccctg agggggatag 30

<210> SEQ ID NO 12
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 12

nnantacagc tgcatccctt cccgctacac 30

<210> SEQ ID NO 13
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(3)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 13

nnnatacagc tgcatccctg actccccgag 30

<210> SEQ ID NO 14
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 14

nncntacagc tgcatccctg tgttgcgcgg 30

<210> SEQ ID NO 15
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(3)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 15

nnnctacagc tgcatccctc tacagcagcg 30

<210> SEQ ID NO 16
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 16

-continued

nngntacagc tgcatccctg tcgcgtcgtt 30

<210> SEQ ID NO 17
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(3)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 17

nnngtacagc tgcatccctc ggagcaacct 30

<210> SEQ ID NO 18
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 18

nntntacagc tgcatccctg gtgaccgtag 30

<210> SEQ ID NO 19
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(3)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 19

nnnttacagc tgcatccctc ccctgtcgga 30

We claim:

1. A system for detecting a sequence of optical signals from each of a plurality of microparticles during a sequence of processing steps, the system comprising:

a planar array of uniformly sized spherical microparticles, wherein the coefficient of variation of the diameters of said microparticles is less than five percent;

an optical train effective to collect and focus the sequence of optical signals from the microparticles, and to record at least one optical characteristic of each microparticle which can be used to determine the approximate center of said microparticle;

an imaging device onto which said signals are focused, effective to generate and record a sequence of digital images of the microparticles, with sufficient resolution for individual microparticles to be distinguished; and

signal tracking means effective to correlate the optical signals from each of the microparticles in each of the sequence of digital images with said center of said microparticle.

2. The system of claim **1**, wherein the microparticles are substantially immobilized in said planar array.

3. The system of claim **1**, wherein said imaging device is a CCD (charge coupled device) camera.

4. The system of claim **1**, wherein said signal tracking means is effective to assign a plurality of pixels to each microparticle in each said image.

* * * * *

EXHIBIT 5

US006654505B2

(12) **United States Patent**
Bridgham et al.

(10) **Patent No.: US 6,654,505 B2**
(45) **Date of Patent: Nov. 25, 2003**

(54) **SYSTEM AND APPARATUS FOR SEQUENTIAL PROCESSING OF ANALYTES**

(75) Inventors: **John Bridgham**, Hillsborough, CA (US); **Kevin Corcoran**, Fremont, CA (US); **George Golda**, El Granada, CA (US); **Michael C. Pallas**, San Bruno, CA (US); **Sydney Brenner**, La Jolla, CA (US)

(73) Assignee: **Lynx Therapeutics, Inc.**, Hayward, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 259 days.

(21) Appl. No.: **09/907,795**

(22) Filed: **Jul. 17, 2001**

(65) **Prior Publication Data**

US 2002/0137052 A1 Sep. 26, 2002

Related U.S. Application Data

(60) Division of application No. 09/424,028, filed as application No. PCT/US98/11224 on May 22, 1998, now Pat. No. 6,406,848, which is a continuation-in-part of application No. 08/862,610, filed on May 23, 1997, now abandoned.

(51) **Int. Cl.**[7] **G06F 15/316**
(52) **U.S. Cl.** **382/278**; 382/129
(58) **Field of Search** 382/278, 288, 382/129, 133, 128; 435/6, 288.3, 297.5, 299.1

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,116,765 A		5/1992	Watanabe	436/165
5,604,097 A		2/1997	Brenner	435/6
5,631,734 A		5/1997	Stern	356/317
5,834,195 A		11/1998	Benkovic	435/6
5,854,684 A		12/1998	Stabile	356/440
5,856,174 A		1/1999	Lipshutz	435/286.5
5,872,623 A		2/1999	Stabile	356/73
5,889,881 A	*	3/1999	MacAulay et al.	382/133
6,026,174 A	*	2/2000	Palcic et al.	382/133
6,031,930 A	*	2/2000	Bacus et al.	435/1.1
6,511,802 B1	*	1/2003	Albrecht et al.	435/6

FOREIGN PATENT DOCUMENTS

EP	0 392 546	10/1990
EP	0 573 098	12/1993

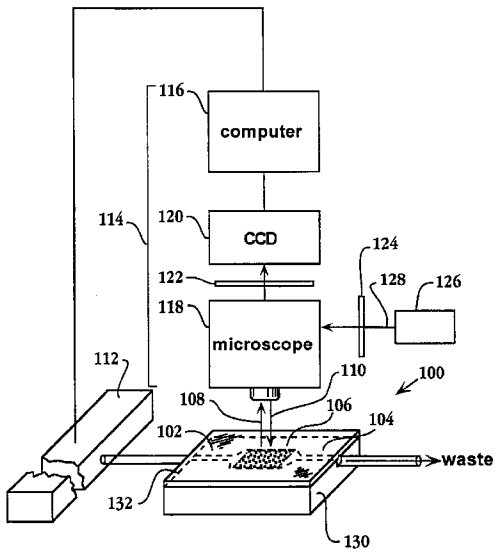
* cited by examiner

Primary Examiner—Yon J. Couso
(74) *Attorney, Agent, or Firm*—Stephen C. Macevicz; Vincent M. Powers; LeeAnn Gorthey

(57) **ABSTRACT**

An apparatus and system are provided for simultaneously analyzing a plurality of analytes anchored to microparticles. Microparticles each having a uniform population of a single kind of analyte attached are disposed as a substantially immobilized planar array inside a flow chamber where steps of an analytical process are carried out by delivering a sequence of processing reagents to the microparticles by a fluidic system under microprocessor control. In response to such process steps, an optical signal is generated at the surface of each microparticle which is characteristic of the interaction between the analyte carried by the microparticle and the delivered processing reagent. The plurality of analytes are simultaneously analyzed by collecting and recording images of the optical signals generated by all the microparticles in the planar array. A key feature of the invention is the correlation of the sequence of optical signals generated by each microparticle in the planar array during the analytical process.

6 Claims, 10 Drawing Sheets



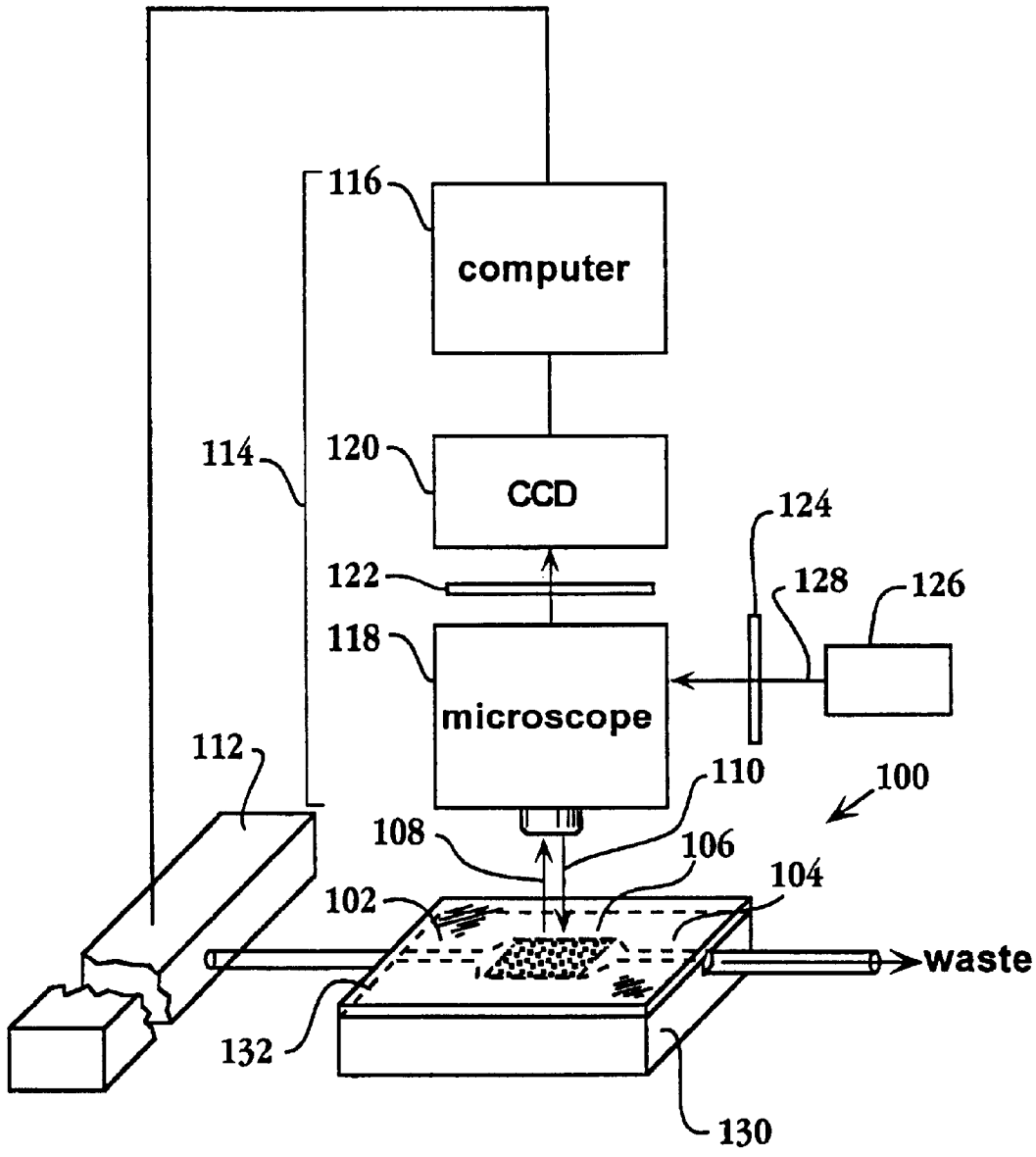

116
computer
114
120
CCD
124
122
128
126
118
microscope
112
110
100
108
106
104
102
waste
132
130

Fig. 1A

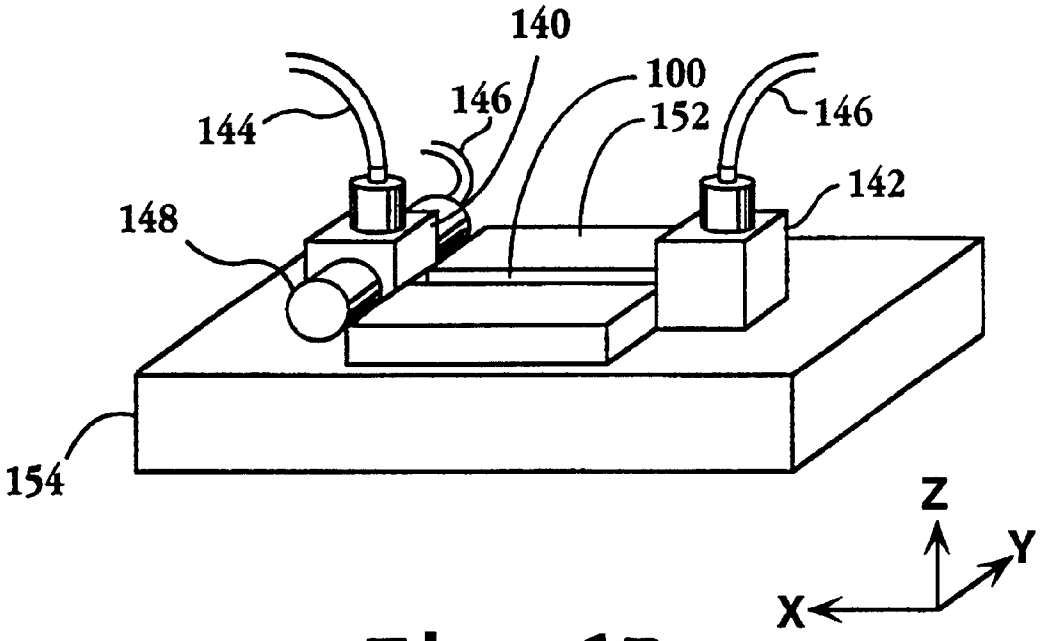


Fig. 1B

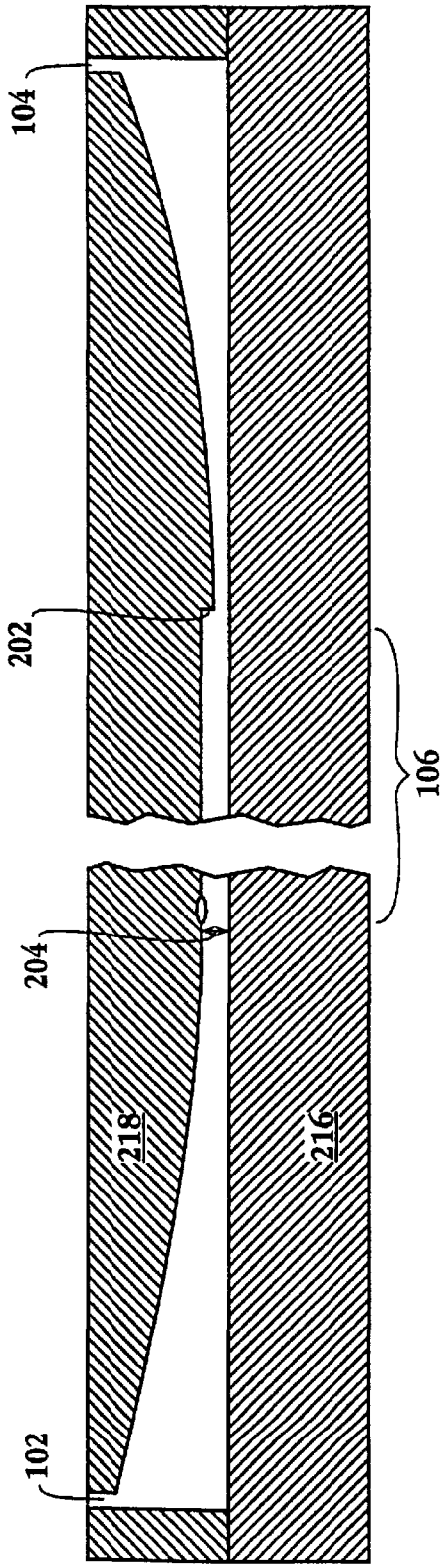

104
202
106
204
218
216
102

Fig. 2A

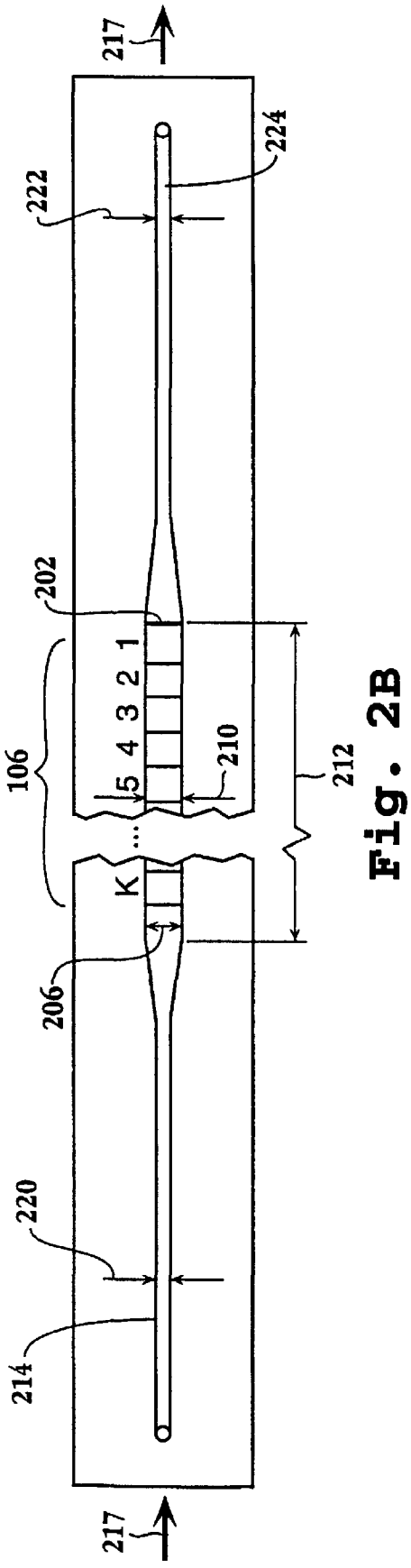

217
222
224
202
1
2
3
4
5
106
210
212
K
206
220
214
217

Fig. 2B

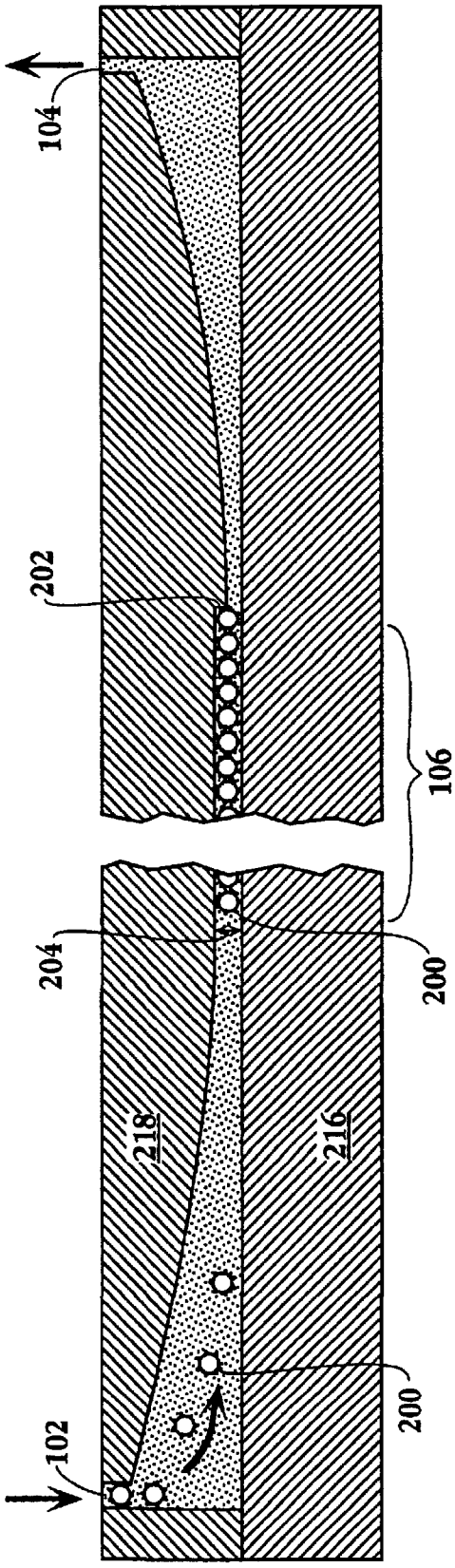


Fig. 2C

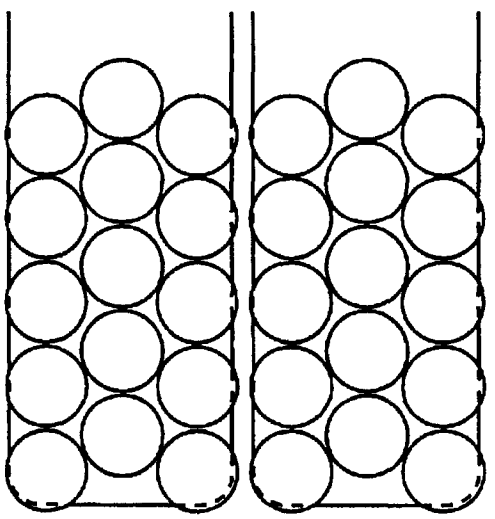


Fig. 3A

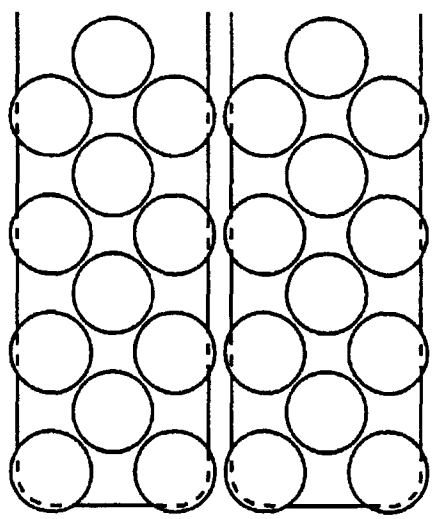


Fig. 3B



Fig. 3C



Fig. 3D

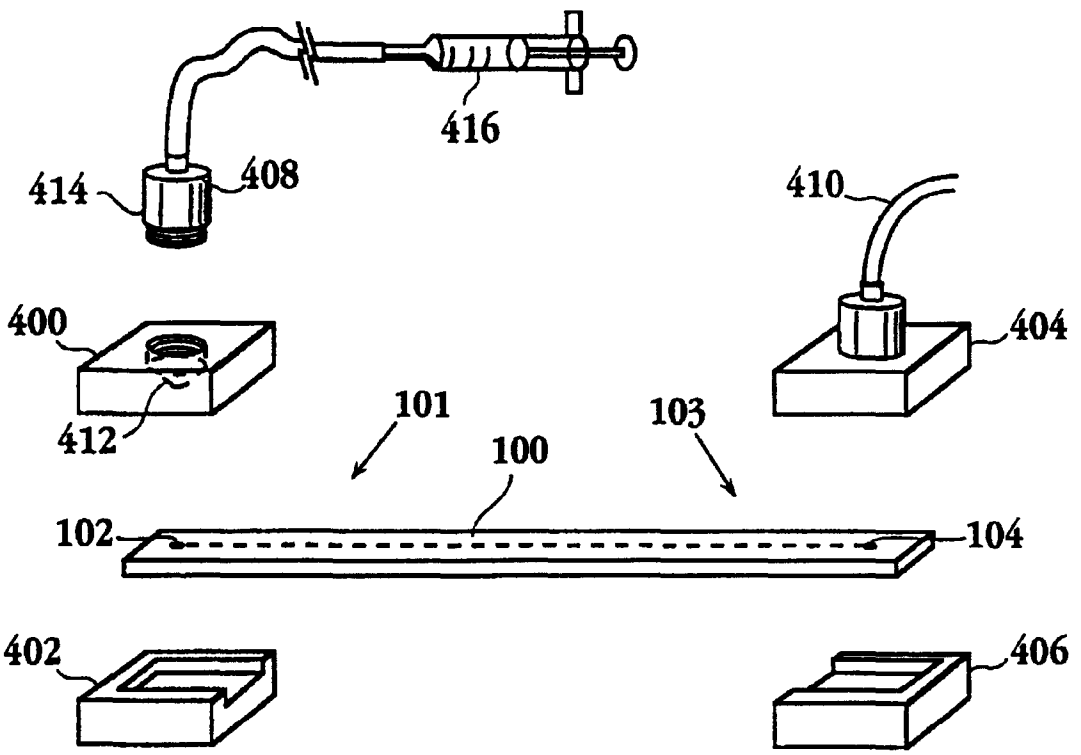


Fig. 4

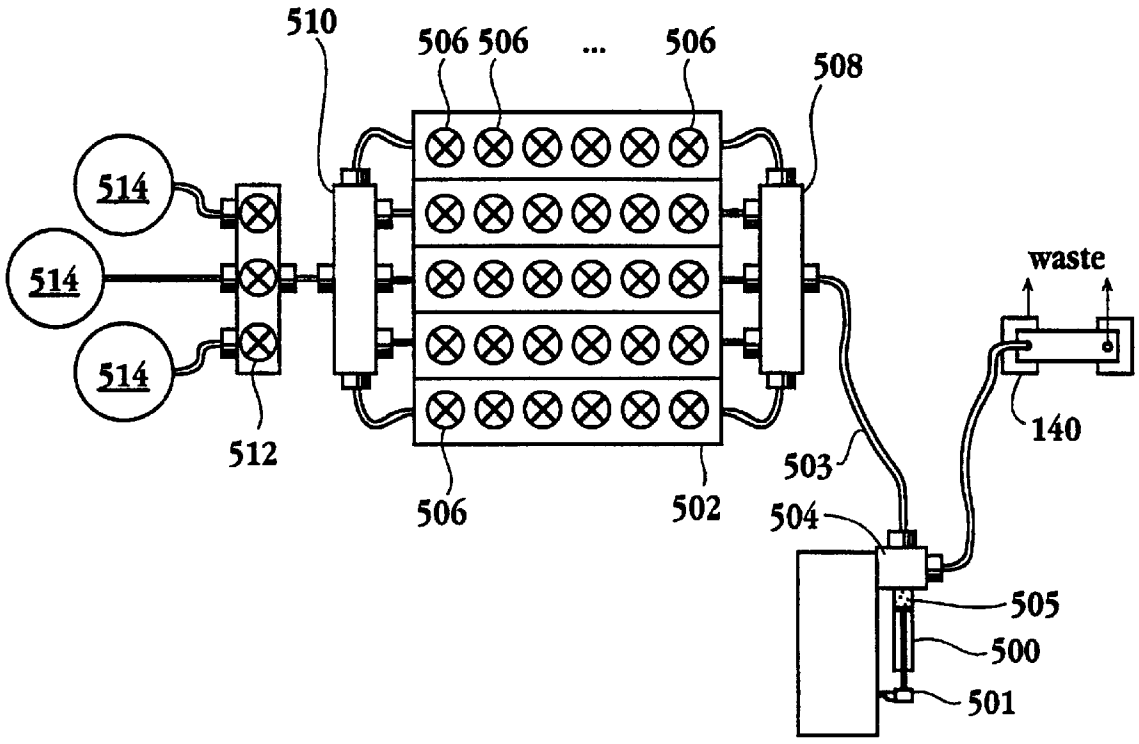

510
506 506 ... 506
508
514
514
514
512
506
502
waste
140
503
504
505
500
501

Fig. 5

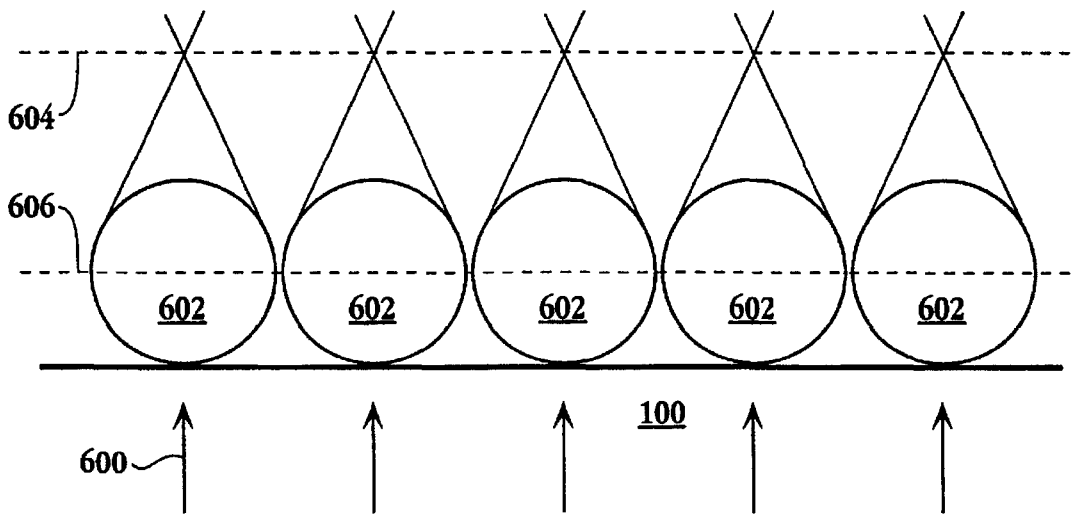

604
606
602
602
602
602
602
100
600

Fig. 6A

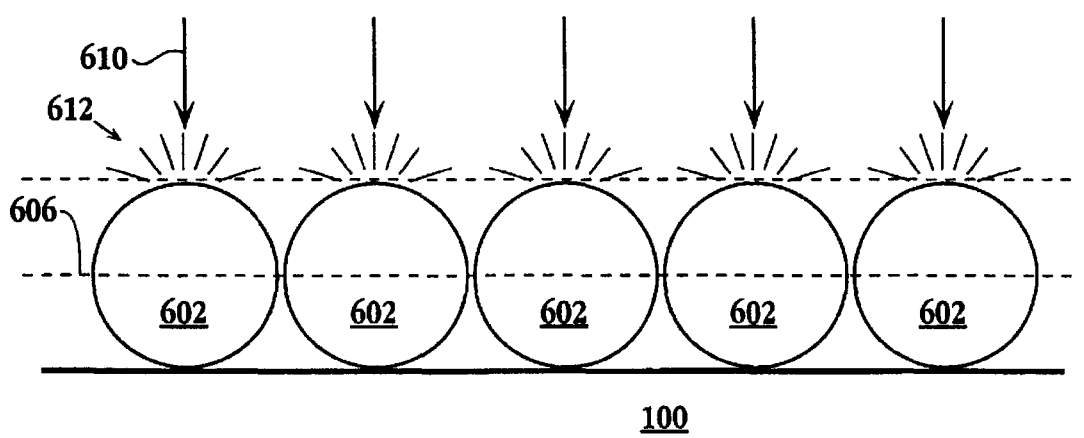

610
612
606
602
602
602
602
602
100

Fig. 6B

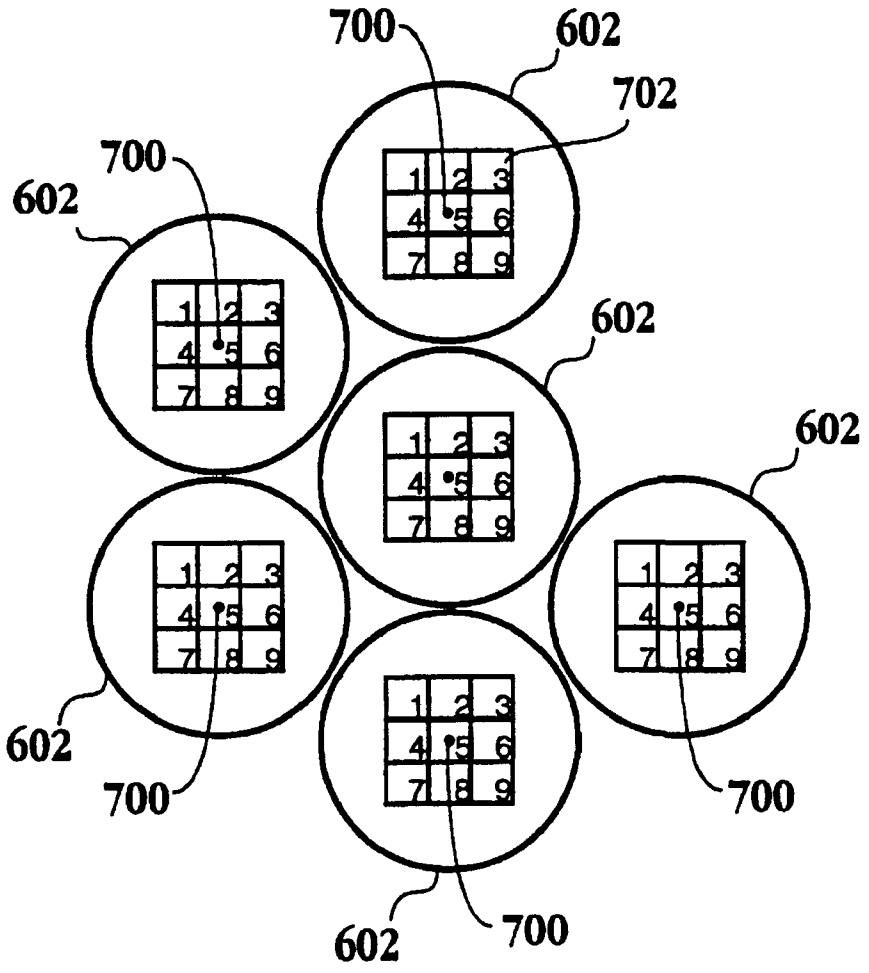


Fig. 7

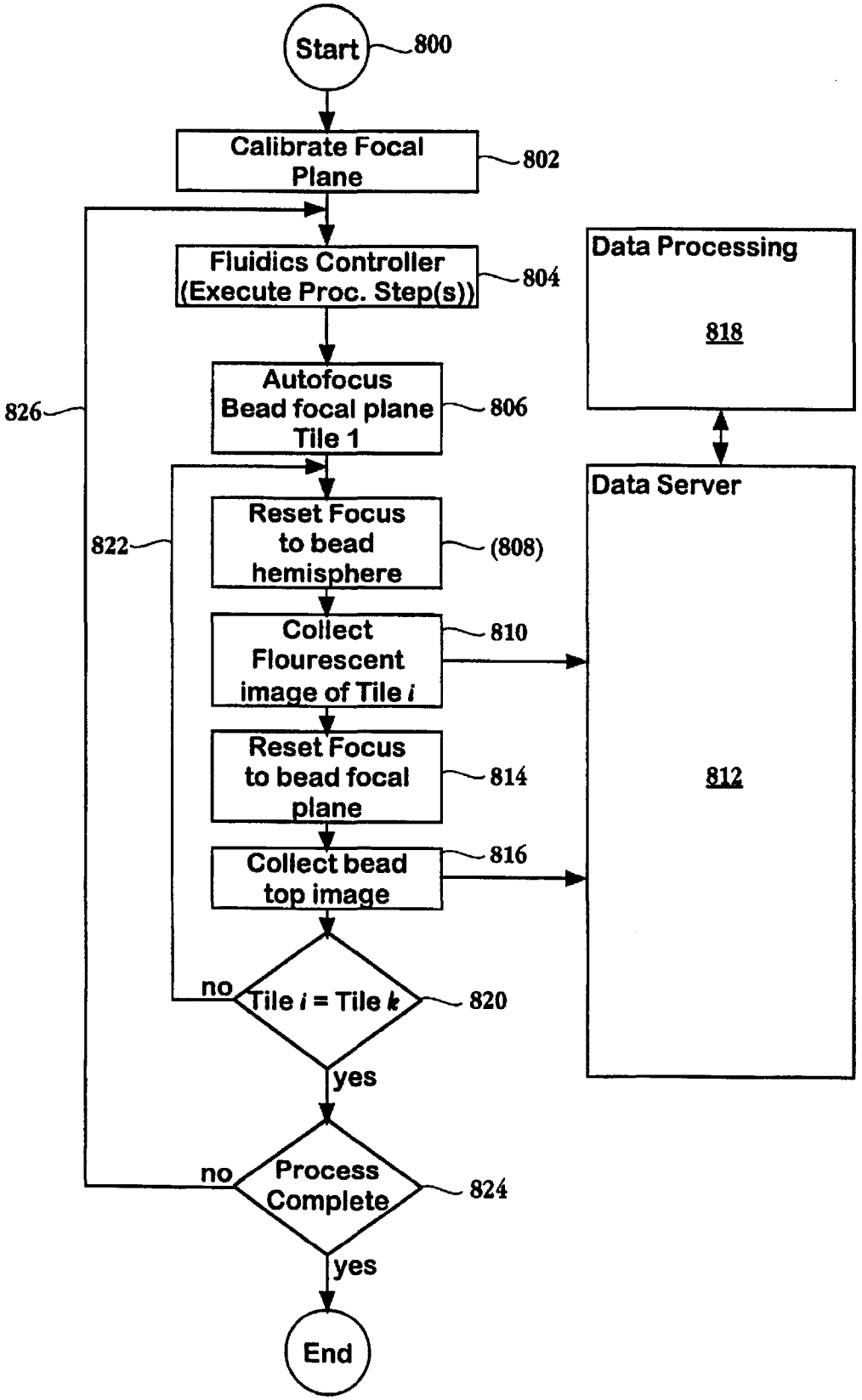

Start
800
Calibrate Focal Plane
802
Fluidics Controller (Execute Proc. Step(s))
804
Autofocus Bead focal plane Tile 1
806
826
Reset Focus to bead hemisphere
(808)
822
Collect Flourescent image of Tile i
810
Reset Focus to bead focal plane
814
Collect bead top image
816
Tile i = Tile k
820
no
yes
Process Complete
824
no
yes
End
Data Processing
818
Data Server
812

Fig. 8

SYSTEM AND APPARATUS FOR SEQUENTIAL PROCESSING OF ANALYTES

This application is a divisional of U.S. patent application Ser. No. 09/424,028, filed Nov. 16, 1999 now U.S. Pat. No. 6,406,848, which is a 371 of PCT/US98/1124, filed May 22, 1998, which is a C-I-P of U.S. patent application Ser. No. 08/862,610, filed May 23, 1997 now abandoned, all of which are incorporated in their entirety herein by reference.

FIELD OF THE INVENTION

The invention relates generally to systems and apparatus for carrying out large scale parallel reactions on solid phase supports, and more particularly, to systems and apparatus for monitoring and carrying out reactions on arrays of microparticles.

BACKGROUND

The desire to understand and analyze complex chemical and biological systems has led to the development of analytical techniques that employ parallelization and miniaturization of analyte processing, e.g. Graber et al, Current Opinion in Biotechnology, 9: 14–18 (1998); Fodor et al, Nature, 364: 555–556 (1993); Meier-Ewert et al, Nature, 361: 375–376 (1993); Taylor et al, Nucleic Acids Research, 25: 3164–3168 (1997); Garner et al, BioTechniques, 14: 112–115 (1993); Lam et al, Nature, 354: 82–84 (1991); Ohlmeyer et al, Proc. Natl. Acad. Sci., 90: 10922–10926 (1993); DeRisi et al, Science, 278: 680–686 (1997); Wodicka et al, Nature Biotechnology, 15: 1359–1367 (1997); and the like.

Many of these techniques employ microparticles for synthesizing analytes or for capturing analytes for subsequent analysis, e.g. Lam et al (cited above); Benkovic et al, International patent application PCT/US95/03355; Gavin et al, International patent application PCT/EP97/02039; Brenner et al, International patent application PCT/US96/09513, and the like. Even though the properties of different types of microparticles can vary widely, microparticles generally facilitate the construction and manipulation of large repertoires of analytes with minimal reagent and/or sample consumption. However, handling and manipulating large numbers of microparticles, e.g. tens to hundreds of thousands, for carrying out specific chemical and/or biochemical analyses gives rise to many difficulties, including whether sufficient signal is generated on individual microparticles for detection, how to track individual microparticles through multiple steps of a process, mechanical strength of microparticles under pressure or flow conditions, the ability to uniformly deliver reagents to microparticles for carrying out steps of an analytical process, whether clumping or other inappropriate interaction of microparticles and/or reagents occurs, the degree to which analytes and/or processing reagents adsorb onto vessel walls, whether protein reagents or analytes denature causing a disruption of reagent distribution and access, whether adjacent microparticles will interact, e.g. to degrade or obscure a signal or to inhibit reagent access, and the like.

In view of these difficulties, it would be desirable to provide a system and apparatus for handling and processing multiple solid phase supports, such as populations of microparticles. It would be especially desirable if such system and apparatus permitted the tracking and analysis of multiple analytes anchored to separate microparticles through a sequence of several processing and/or analysis steps.

SUMMARY OF THE INVENTION

Accordingly, objects of our invention include, but are not limited to, providing a system and apparatus for sequentially

delivering reagents to a population of analytes anchored to separate microparticles; providing an apparatus for simultaneously monitoring the interactions of processing reagents and analytes on the surfaces of microparticles disposed in a planar array; providing an apparatus for detecting optical signals generated by, or as the result of, interactions of processing reagents and analytes on the surfaces of microparticles disposed in a planar array; providing an apparatus for detecting pluralities of optical signals, each such plurality being generated at the surface of the same microparticle as a result of interactions between processing reagents and an analyte anchored to the surface of such microparticle; providing an apparatus for simultaneously tracking the positions of individual microparticles in a population of microparticles disposed in a flow chamber as a closely packed planar array; and providing a system and apparatus for simultaneously analyzing the nucleotide sequences of a population of polynucleotides anchored to microparticles disposed in a planar array in a flow chamber.

Our invention achieves these and other objects with an apparatus comprising a flow chamber for disposing a population of microparticles in a planar array; fluidic means for sequentially delivering processing reagents from one or more reagent reservoirs to the flow chamber; and detection means for detecting a sequence of optical signals from each of the microparticles of the population. Preferably, the sequences of optical signals are generated as a result of a multi-step analytical process, such as nucleic acid sequence analysis.

In one aspect, the invention provides a system for simultaneously monitoring a population of analytes which includes the apparatus of the invention, microparticles carrying the analytes, and software means for processing images of, and/or optical signals generated by, the microparticles when disposed in a planar array. Preferably, the flow chamber includes constraining means for restricting the movement of microparticles during cycles of reagent delivery.

In another aspect, the invention includes a system for simultaneously analyzing the nucleotide sequences of a population of polynucleotides. Copies of each kind of polynucleotide in the population are sorted onto and anchored to one or more microparticles so that a population of loaded microparticles is formed. Loaded microparticles are disposed in a planar array in a flow chamber through which processing reagents are sequentially delivered to the loaded microparticles from one or more reagent reservoirs by a fluidic means. Optical signals generated by, or produced as a result of, the interaction of processing reagents and polynucleotides on the microparticles are imaged by a detection means. Preferably, when analysis include determining the nucleotide sequence of a portion of each polynucleotide on the different microparticles, massively parallel signature sequencing (MPSS) analysis is employed, e.g. as described in Albrecht et al, International patent application PCT/US97/09472.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1***a* is a schematic representation of a flow chamber and fluidics and detection systems for observing a planar array of microparticles loaded with analyte molecules, such as cDNA molecules for sequencing.

FIG. **1***b* is a schematic of a preferred holder for a flow chamber.

FIG. **2***a* is bilateral cut away view of a flow chamber.

FIG. **2***b* is a top view of a flow chamber.

FIG. **2***c* is an illustration of microparticles being loaded into a flow chamber.

FIGS. **3***a* through **3***d* schematically illustrate microparticle constraining means for a flow chamber.

FIG. **4** is a schematic representation of a device for loading microparticles into a flow chamber.

FIG. **5** is a schematic representation of a fluidics system for use with the invention.

FIGS. **6***a* and **6***b* schematically illustrate top-lighting and back-lighting approaches for determining microparticle centers in an array.

FIG. **7** schematically illustrates the assignment of pixels to microparticles for data processing.

FIG. **8** is a flow chart summarizing operation of the system of the invention.

DEFINITIONS

"Complement" or "tag complement" as used herein in reference to oligonucleotide tags refers to an oligonucleotide to which a oligonucleotide tag specifically hybridizes to form a perfectly matched duplex or triplex. In embodiments where specific hybridization results in a triplex, the oligonucleotide tag may be selected to be either double stranded or single stranded. Thus, where triplexes are formed, the term "complement" is meant to encompass either a double stranded complement of a single stranded oligonucleotide tag or a single stranded complement of a double stranded oligonucleotide tag.

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, anomeric forms thereof, peptide nucleic acids (PNAs), and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g. 3–4, to several tens of monomeric units, e.g. 40–60. Whenever an oligonucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, unless otherwise-noted. Usually oligonucleotides of the invention comprise the four natural nucleotides; however, they may also comprise non-natural nucleotide analogs. It is clear to those skilled in the art when oligonucleotides having natural or non-natural nucleotides may be employed, e.g. where processing by enzymes is called for, usually oligonucleotides consisting of natural nucleotides are required.

"Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one other such that every nucleotide in each strand undergoes Watson-Crick basepairing with a nucleotide in the other strand. The term also comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, and the like, that may be employed. In reference to a triplex, the term means that the triplex consists of a perfectly matched duplex and a third strand in which every nucleotide undergoes Hoogsteen or reverse Hoogsteen association with a basepair of the perfectly matched duplex. Conversely, a "mismatch" in a duplex between a tag and an oligonucleotide means that a pair or triplet of nucleotides in the duplex or triplex fails to undergo Watson-Crick and/or Hoogsteen and/or reverse Hoogsteen bonding.

As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e. g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543–584 (1990), or the like, with the only proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like.

As used herein "sequence determination" or "determining a nucleotide sequence" in reference to polynucleotides includes determination of partial as well as full sequence information of the polynucleotide. That is, the term includes sequence comparisons, fingerprinting, and like levels of information about a target polynucleotide, as well as the express identification and ordering of nucleosides, usually each nucleoside, in a target polynucleotide. The term also includes the determination of the identification, ordering, and locations of one, two, or three of the four types of nucleotides within a target polynucleotide. For example, in some embodiments sequence determination may be effected by identifying the ordering and locations of a single type of nucleotide, e.g. cytosines, within the target polynucleotide "CATCGC . . . " so that its sequence is represented as a binary code, e.g. "100101 . . . " for "C-(not C)-(not C)-C-(not C)-C . . . " and the like.

As used herein, the term "complexity" in reference to a population of polynucleotides means the number of different species of molecule present in the population.

DETAILED DESCRIPTION OF THE INVENTION

The system and apparatus of the invention is particularly applicable to the analysis of molecules that can be anchored in populations of duplicate copies to particulate solid phase supports. That is, in accordance with the invention, each analyte of a population is present on at least one microparticle in a quantity sufficient for the type of analysis being performed. For example, if combinatorially synthesized peptides on the microparticles are screened against a soluble receptor protein for detecting those that form stable complexes, the number of peptides available for binding on the surface of the microparticles must be large enough to generate a detectable signal when a binding event occurs. Of course, many additional factors well known in the art will present additional design constraints, such as the nature of the system for generating optical signals, the concentration of receptors, pH, salt concentration, the density and accessibility of the peptides on the microparticle surface, the solvent system employed, and the like. Analyte populations particularly relevant for use with the present apparatus include combinatorial libraries synthesized on microparticle supports, e.g. as disclosed in Lam et al, Chem. Rev., 97: 411–448 (1997); or Dower et al, U.S. Pat. No. 5,708,153, and polynucleotide libraries sorted onto microparticle supports, e.g. as disclosed in Brenner (cited above).

FIG. **1***a* is a schematic representation of an embodiment of the invention for detecting fluorescent signals. Flow chamber (**100**) having inlet (**102**), outlet (**104**) and planar

cavity (**106**) holds microparticles in a planar array from which optical signals (**108**) generated by analytes and/or reactants on microparticles can be collected and imaged. Flow chamber (**100**) is operationally associated with fluidic system (**112**) and detection system (**114**), so that delivery of fluids and collection of signals is under control of computer (**116**). Preferably, optical signals are collected by microscope (**118**) and are imaged onto a solid state imaging device, such as charge-coupled device (CCD) (**120**) which is capable of generating a digital image of the physical image of the microparticle array with sufficient resolution for individual microparticles to be distinguished. For fluorescent signals, detection system (**114**) usually includes appropriate bandpass filter (**122**) for optical signal (**108**), bandpass filter (**124**) for excitation beam (**128**) generated by light source (**126**), and other standard components. As illustrated, a conventional fluorescence microscope is preferred which is configured for epiillumination. There is a great deal of guidance in the art for selecting appropriate fluorescence microscopes, e.g. Wang and Taylor, editors, Fluroescence Microscopy of Living Cells in Culture, Parts A and B, Methods in Cell Biology, Vols. 29 and 30 (Academic Press, New York, 1989).

A key feature of the invention is flow chamber (**100**). Body (**130**) of flow chamber (**100**) preferably comprised inlet (**102**), outlet (**104**) and planar cavity (**106**) which are formed by standard micromachining techniques, e.g. Ekstrom et al, International patent application PCT/SE91/00327; Brown, U.S. Pat. No. 4,911,782; Harrison et al, Anal. Chem. 64: 1926–1932 (1992); and the like. Transparent plate (**132**) is sealingly attached to body (**130**) to form an operational flow chamber (**100**). Body (**130**) may be constructed from any of several different materials including glass, silicon, polyethylene, polyester, teflon, other plastics, and the like. Preferably, transparent plate (**132**) is glass or quartz; and, when body (**130**) and transparent plate (**132**) are glass or silicon, transparent plate (**132**) is preferably attached to body (**130**) by anodic bonding, e.g. Pomerantz, U.S. Pat. No. 3,397,279. Key functions of the flow chamber include i) holding a population of microparticles in a substantially immobilized planar array, or monolayer, during a sequence of processing steps, ii) ensuring that processing reagents can access each microparticle during each step of a process, and iii) minimizing processing reagent usage. The degree of immobilization required may vary among different embodiments. Generally, more movement of microparticles within a planar array increases the computational and measurement burden of tracking positions of microparticles by image processing software. Design trade-offs therefore exist between the use of image processing software and the use of physical and/or chemical means for constraining microparticle movement. Preferably, physical and/or chemical means are employed to constrain microparticle movement within the planar array of microparticles in flow chamber (**100**). Such means are referred to herein as "movement constraining means." Most preferably, physical, or mechanical, movement constraining means are employed.

Preferably, microparticles are disposed in flow chamber (**100**) in a closely packed planar array. As used herein, "closely packed" in reference to a planar array means either that the number of microparticles per unit area of a planar array is at least eighty percent of the number of microparticles in a hexagonal array of equal area, or that the average distance between centers of adjacent microparticles is less than two microparticle diameters. As used herein, a "hexagonal" array of microparticles means a planar array of microparticles in which every microparticle in the array contacts at least six other adjacent microparticles, as shown in FIG. **3***a*.

Additions features of flow chamber (**100**) of a preferred embodiment are illustrated in FIGS. **2***a* through **2***c*. FIG. **2***a* is a cross sectional view along a longitudinal plane that bisects flow chamber (**100**). The same view, in a more abstracted rendition, is shown in FIG. **2***c*. In both Figures, inlet (**102**) fluidly communicates with planar cavity (**106**) and outlet (**104**). Microparticles (**200**) carrying analytes enter inlet (**102**) and are carried by a suspending buffer to planar cavity (**106**) where they become packed against dam (**202**) which prevents the microparticles from exiting the flow chamber through outlet (**104**). Structurally, dam (**202**) may be formed by a sudden reduction of the vertical dimension of planar cavity (**106**). Preferably, vertical dimension (**204**) of planar cavity (**106**) is selected so that microparticles (**200**) are constrained to a plane, i.e. a monolayer, when they pack against dam (**202**). More preferably, vertical dimension (**204**) is selected to be between about 120 to 150 percent of the diameter of the microparticles employed. For example, when microparticles are employed that have diameters of 5 μm, vertical dimension (**204**) may be 7 μm. Magnetic microparticles may be constrained to a plane and constrained from movement by applying a magnetic field so that the microparticles are attracted to the ceiling or to the floor of planar cavity (**106**). Width (**206**) of planar cavity (**106**) is not a critical dimension; however, for convenience and efficiency, width (**206**) may be selected to correspond to the dimensions of the signal collection region of detection system (**114**). Such regions labeled l through k in FIG. **2***b* are referred to herein as "tiles." That is, the region of planar cavity (**106**) occupied by microparticles may be divided into non-overlapping areas, referred to as "tiles," that cover the entire occupied region. FIG. **2***b,* which is a top view of the flow chamber of FIG. **2***a,* also shows inlet (**102**), planar cavity (**106**), dam (202), and outlet (**104**) that lie in sequence along axis (**217**) of flow chamber (**100**).

Many movement constraining means may be selected for use with the flow chamber, either alone or in combination. Such means include loading microparticles with trace amounts of a chemically reactive species which may be activated and cross-linked; providing physical, or mechanical structures, such as ridges, within the flow chamber; providing magnetically responsive microparticles which may be immobilized by an external magnetic field; providing a second population of microparticles that are loaded into a flow chamber after the analyte-containing population, which forces the analyte-containing population against dam (**202**); and the like. Exemplary chemically reactive species for use with nucleic acid analytes are disclosed in Summerton et al, U.S. Pat. No. 4,123,610; Gamper et al, J. Mol. Biol., 197: 349–362 (1987); Hearst, Ann. Rev. Phys. Chem. 39: 291–315 (1988); Pieles et al, Nucleic Acids Research, 17: 8967–8978 (1989); and the like.

Preferably, microparticle movement is constrained by providing a flow chamber with planar cavity (**106**) containing a plurality of ridges running parallel to axis (**217**) of the flow chamber, i.e. parallel to the direction of reagent flow, so that microparticles are arranged into rows, which may be single-file, or several microparticles wide, as shown in FIGS. **3***a* and **3***b*. The particular selection may depend on several factors, including the degree of immobilization desired, constraints imposed by the fabrication technique used to construct the flow chamber, the amount of reagent access desired, the degree to which flow resistance or back-pressure can be tolerated, and the like. FIGS. **3***a* and **3***b* illustrate two possible distances between parallel ridges. In FIG. **3***a,* the distance is selected to permit maximal packing of microparticles into a hexagonal array, and in FIG. **3***b,* the

distance is selected for less efficient packing, but for increased reagent access to microparticle surfaces. FIGS. **3***c* and **3***d* are axial views of the flow chamber showing the microparticle arrangements of FIGS. **3***a* and **3***b,* respectively.

In some embodiments, such as those employing enzymatic processes, the inner surfaces of flow chamber **(100)** may be passivated, that is, treated to render such surfaces inert and/or non-adsorbing with respect to enzymes. The type of treatment depends on the sensitivity of the enzymes used in the process, and their affinity for the surfaces. Surface treatments include silanization, e.g. with commercially available reagents (Pierce, Rockford, Ill.); and/or adsorption of various blocking polymers, such as poly-a-alanine, polyglycine, polyadenylic acid, polymaleimide, polyvinylpyrrolidone, or the like, e.g. Shoffner et al, Nucleic Acids Research, 24: 375–379 (1996). Preferably, glass inner surfaces of flow chamber **(100)** are covalently coated with a neutral coating, such as allyl methacrylate, using the technique disclosed in Sandoval et al, U.S. Pat. No. 5,326,738, which is incorporated by reference.

FIG. **1***b* illustrates flow chamber **(100)** mounted between holders **(140)** and **(142)** which sealingly connect inlet **(102)** to inlet tubing **(144)** and outlet **(104)** to outlet tubing **(146)**, respectively. Preferably, holder **(140)** contains a rotary valve (not shown) operated by actuator **(148)** that shunts fluid flowing through inlet tubing **(144)** to inlet **(102)** or to waste line **(150)**. Such a valve minimizes the amount of process reagent from a previous step that must be passed through flow chamber **(100)** prior to the initiation of the next process step. That is, such a rotary valve permits reagent in inlet tubing **(144)** to be shunted to waste and replaced by processing reagent required for the next step in the process being executed. Preferably, for use in DNA analysis, peltier block **(152)** is employed to control temperature in flow chamber **(100)** and the entire assembly including flow chamber **(100)** and peltier block **(152)** is mounted on xyz-stage **(154)** which is under control of computer **(116)**.

Preferably, microparticles are loaded into flow chamber **(100)** prior to attachment of holders **(140)** and **(142)** and the initiation of processing steps. FIG. **4** illustrates a microparticle loader for loading microparticles into flow chamber **(100)**. Flow chamber **(100)** is mounted between holders **(400)**, **(402)**, **(404)**, and **(406)**. Holders **(400)** and **(402)** sealingly clamp onto the inlet end **(101)** of flow chamber **(100)** and holders **(404)** and **(406)** sealingly clamp onto the outlet end **(103)** of flow chamber **(100)** so that inlet tubing **(408)** is in fluid communication with outlet tubing **(410)** when the microparticle loader is assembled. Inlet tubing **(408)** is connected to syringe **(416)** which is used to drive fluid through flow chamber **(100)**. Holder **(400)** is constructed to have conical passage **(412)** which narrows to match the diameter of inlet **(102)** of flow chamber **(100)**. After assembly of holders **(400)**, **(402)**, **(404)**, and **(406)** a suspension of microparticles is placed in the conical passage after which fitting **(414)** is sealingly connected to holder **(400)**. Fluid pressure and flow generated by syringe **(416)** then drives the microparticles into planar cavity **(106)** and against dam **(202)**. In a preferred embodiment which employs 5 μm diameter GMA microparticles carrying DNA, approximately 500 thousand microparticles are loaded into flow chamber **(100)** by placing 5 μL of a 100 thousand microparticle/μL solution (TE buffer, pH 8.0, Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989)) in conical passage **(412)**, attaching fitting **(414)**, and using syringe **(416)** to drive the microparticles through inlet **(102)** and into planar cavity **(106)**. After loading, holders **(400)**, **(402)**, **(404)**, and **(406)** are removed from flow chamber **(100)**, which is then mounted on the apparatus as shown in FIG. **1***b*.

Preferably, process reagents are delivered to flow chamber **(100)** by the fluidic system illustrated in FIG. **5** which has the capacity to handle many different reagents for complex analytical processes. In the illustrated embodiment, which is used in connection with DNA sequencing, the fluidics system may accommodate up to 38 reagents, including wash buffers, rinses, enzymes, hybridization probes, adaptors, and the like. Preferably, the function of the fluidics system is the sequential metering of selected processing reagents to flow chamber **(100)**. Inlet **(102)** of flow chamber **(100)** is sealingly connected to holder **(140)** which contains rotary valve (actuator shown as **148)** (not shown in FIG. **5)**. The function of the rotary valve is described above. A variety of means may be employed for moving processing reagents from reservoirs, through tubing, and into flow chamber **(100)**, including gravity feed, pressure feed, and pumps, e.g. peristaltic, syringe, and the like. Preferably, common syringe pump **(500)** is employed for removing predetermined amounts processing reagents from reservoirs and for forcing such reagents through flow chamber **(100)** at a predetermined flow rate. Under control of computer **(116)**, pump **(500)** in operational association with valve block **(502)** and rotary valve **(504)** removes a predetermined amount of processing reagent from a selected reservoir by siphoning reagent out of the reservoir on the out-stroke of plunger **(501)** of pump **(500)**. On the in-stroke of plunger **(501)**, rotary valve **(504)** directs processing reagent from tubing **(503)** to reservoir **(505)** of pump **(500)**. On the out-stroke of plunger **(501)**, state of rotary valve **(504)** is changed to direct processing reagent from reservoir **(505)** to inlet tubing **(144)**. Tubing **(503)** connects rotary valve **(504)** with manifold **(508)** which, in turn, is connected to a plurality (five shown) of banks of zero dead volume valves **(506)**. Zero dead volume valves **(506)** connect individual reservoirs holding processing reagents to a common passageway (not shown in FIG. **5)** that runs through each of the banks of valves connecting to manifold **(508)**.

A preferred zero dead volume valve is described in U.S. Pat. Nos. 4,558, 845 and 4,703,913, which are incorporated by reference. Process reagents from reservoirs **(514)** are distributed to the banks of dead volume valves by way of manifold **(510)**. Alternative valve blocks for controlling delivery of process reagents to flow chamber **(100)** include the valve matrix disclosed in U.S. Pat. No. 5,203,368.

An important feature of detection means **(114)** of the invention is the ability to keep track of individual microparticles through multiple process steps and/or cycles. In connection with such tracking, detection means **(114)** periodically records optical characteristics of individual microparticles that provide a close approximation microparticle centers. Preferably, when trans-illumination, or "back lighting" of flow chamber **(100)** is possible, the optical characteristic is the focused back light from the microparticles. That is, in reference to FIG. **6***a,* back light **(600)** passes vertically through flow chamber **(100)** where it is focused by microparticles **(602)** onto focal plane **(604)**. The image of focal plane **(604)** in this configuration appears as a field of bright points, where each point is located at the approximate center of its corresponding microparticle. In an epillumination system, light from above flow chamber **(100)**, i.e. "top light **(610)**," is directed from a vertical direction onto microparticles **(602)** where it scatters from the top surface of the microparticles. In this configuration, the optical characteristic is the scatter center of a microparticle. Thus, an

image is collected from the plane containing scatter centers (**612**) resulting from such top lighting. As with focused back lighting, the image of the scatter centers provides a convenient way to readily determine the approximate centers of the microparticles.

In the preferred image processing approach, once microparticle centers (**700**) are determined, pixels (**702**) are assigned for determining characteristics, e.g. intensity, of an optical signal generated at each microparticle (**602**). The size of microparticle (**602**) and pixel area determine how many pixels are assigned to each microparticle. In making such an assignment, important factors include the degree to which the calculated center of a microparticle (as described above) is likely to deviate from the geometric center, the extent to which optical signal collected from the edge of an image contains spurious information (e.g. signal from an overlapping or adjacent microparticle), the uniformity of microparticle diameter and shape, and the like. In the preferred apparatus of the invention, 5 μm diameter microparticles are employed and the pixel dimensions of the CCD detector are about 0.9 μm×0.9 μm. Thus, nine pixels fit easily within the interior of a microparticle image with a margin of at least about 1 μm between any pixel and the edge of the microparticle image. In the preferred embodiment, an initial pixel is assigned which encloses the computed center of a microparticle, e.g. pixel "5" in FIG. **7**. Thereafter, additional pixels are assigned, usually the immediately adjacent pixels. Preferably, the value of the optical signal generated by a process at the surface of a microparticle is the average value of the optical signals collected by pixels assigned to that microparticle.

The general operation of the system of the preferred embodiment is summarized by the flow chart of FIG. **8**. At the start (**800**) of an analysis, microparticles with anchored analytes have been loaded into flow chamber (**100**) which has been operationally mounted in holders **140** and **142**. The initial operation is the calibration of the microparticle focal plane (**802**). That is, the vertical, or "z", position of the xyz-stage is determined which optimizes the focus of either the scatter centers of the microparticles, i.e. the microparticle tops for top-lighting, or the focus points of the microparticles for back-lighting. The optimization is carried out by a conventional autofocusing algorithm which provides an image contrast function constructed from a predetermined sample of regions within a collected image. For example, the contrast function may be evaluated iteratively for sequence of z-positions so that the differences of successive values of the contrast function can be determined. These are tested until a difference is found below a predetermined threshold, which is taken as the maximum of the contrast function. Focal plane location is taken as the z position which maximizes the image contrast function. Such calibration is carried out for each tile, if more than one tile is employed, so that a correction table is constructed of changes in stage setting values with respect to the settings of the first tile that are required to bring the system into focus upon translation to subsequent tiles. These values are stored by computer (**116**).

After calibration, process steps are initiated (**804**) by way of a fluidics controller operationally associated with computer (**116**). After process steps (**804**) are completed, stage settings are adjusted to place the first tile into focus using the autofocus algorithm (**806**), which places the focal plane of the microscope objective approximately at the tops of the microparticles. Stage settings are then adjusted (**808**) to bring the focal plane of the microscope objective to the approximate centers of the microparticles, as illustrated (**606**) in FIGS. **6***a* and **6***b*. The amount of stage movement in this re-focusing depends on the diameter of the microparticles being used. After appropriate selection of filters (**124**) and (**122**), a fluorescent image of the first tile is collected (**810**) and transferred to data server (**812**). Fluorescent images are collected on the plane of the microparticle centers because of imperfections in the planar array. That is, microparticles in planar cavity (**106**) do not lie in a perfect planar array for a variety of reasons. For example, some microparticles are elevated above others as a result of packing into the flow chamber, there is some variability in the size and shape of the microparticles; and, the floor of planar cavity (**106**) may be uneven. After the fluorescent image is collected, the focal plane of the microscope objective is returned (**814**) to the microparticle focal plane, where another image is collected (**816**) for the purpose of computing microparticle centers as described above. The image of microparticle centers is transferred to data server (**812**) where data processor (**818**) assigns pixels of the fluorescent image to each microparticle center, as described above. After the image of microparticle centers is collected (**816**), the stage is moved so that an image of the next tile can be collected (**822**). If there are no further tiles of microparticles (**820**), then the next steps and/or cycles of the process are executed (**826**). If there are no further process steps (**824**), then the process is complete and the apparatus is placed in a holding mode.

Optical signals collected in the course of analysis may be generated by a variety of mechanisms, including absorption and fluorescence, chemiluminescence, electrochemiluminescence, or bioluminescence emission. Extensive guidance is available for selecting appropriate optical signaling means, e.g. Kessler, editor, Nonradioactive Labeling and Detection of Biomolecules (Springer-Verlag, Berlin); Keller and Manak, DNA Probes, Second Edition (Stockton Press, New York, 1993); and the like. Preferably, optical signals generated in processing steps are fluorescence emissions.

Microparticles

An important feature of the system of the invention is the use of microparticles for carrying analytes. A variety of microparticles may be employed depending on particular applications. Generally, microparticles must consist of a material compatible with the reagents and chemistry of the process steps being carried out and microparticle must be substantially mechanically rigid so that they retain their shape and size during process steps. Preferably, as used herein, the term "substantially mechanically rigid" means that microparticles neither swell nor contract by more than ten percent (as measure by diameter) in any process solvent or reagent. Preferably, microparticles are microspheres of uniform size, i.e. microparticles are monodisperse. More preferably, the diameters of spherical microparticles have a coefficient of variation less than five percent, and most preferably, less than two percent. Microparticle diameters are in the range of from 0.1 μm to 100 μm. Preferably, microparticle diameters range from 1 μm to 20 μm. Most preferably, microparticle diameters are in the range of 1 to 5 μm. Suitable microparticle materials include inorganic support materials such as glass, e.g. controlled-pore glass, Balltoni beads; silica, zirconia, and the like, e.g. Weetall, Methods in Enzymology, 44: 134–148 (1976); and organic support materials such as highly cross-linked polystyrene, polyacrylate, polymethylmethacrylate, glycidylmethacrylate (GMA), Dynabeads (Dynal, Oslo, Norway), and the like, Rembaum et al, U.S. Pat. No. 4,046,720; Hodge and

Sherrington, editors, pages 435–456, Polymer-supported Reactions in Organic Synthesis (Wiley & Sons, New York, 1980); Andrus et al, U.S. Pat. No. 5,047,524; and the like.

Attaching Identical Conies of Polynucleotides to Microparticles by Solid Phase Cloning

In a preferred embodiment of the invention, identical copies of polynucleotides from a population are anchored to separate microparticles by solid phase cloning, i.e. the use of oligonucleotide tags for sorting polynucleotides onto microparticles such that only the same kind of polynucleotide will be attached to the same microparticle, e.g. Brenner, U.S. Pat. No. 5,604,097, which is incorporated by reference. This condition is accomplished by taking a sample of the full ensemble of tag-polynucleotide conjugates. (It is acceptable that identical polynucleotides have different tags, as it merely results in the same polynucleotide being operated on or analyzed twice in two different locations.) Such sampling can be carried out either overtly—for example, by taking a small volume from a larger mixture—after the tags have been attached to the polynucleotides, it can be carried out inherently as a secondary effect of the techniques used to process the polynucleotides and tags, or sampling can be carried out both overtly and as an inherent part of processing steps.

Oligonucleotide tags for use with the invention are members of a minimally cross-hybridizing set of oligonucleotides. The sequences of oligonucleotides of such a set differ from the sequences of every other member of the same set by at least two nucleotides. Thus, each member of such a set cannot form a duplex (or triplex) with the complement of any other member with less than two mismatches. Complements of oligonucleotide tags of the invention, referred to herein as "tag complements," may comprise natural nucleotides or non-natural nucleotide analogs. Tag complements are attached to microparticles.

Minimally cross-hybridizing sets of oligonucleotide tags and tag complements may be synthesized either combinatorially or individually depending on the size of the set desired and the degree to which cross-hybridization is sought to be minimized (or stated another way, the degree to which specificity is sought to be enhanced). For example, a minimally cross-hybridizing set may consist of a set of individually synthesized 10-mer sequences that differ from each other by at least 4 nucleotides, such set having a maximum size of 332 (when composed of 3 kinds of nucleotides and counted using a computer program such as disclosed in Appendix Ic of International patent application PCT/US96/09513). Alternatively, a minimally cross-hybridizing set of oligonucleotide tags may also be assembled combinatorially from subunits which themselves are selected from a minimally cross-hybridizing set. For example, a set of minimally cross-hybridizing 12-mers differing from one another by at least three nucleotides may be synthesized by assembling 3 subunits selected from a set of minimally cross-hybridizing 4-mers that each differ from one another by three nucleotides. Such an embodiment gives a maximally sized set of 9^3, or 729, 12-mers, "9" is number of oligonucleotides generated by the computer program of Appendix Ia of International patent application PCT/US96/09513, which assumes, as with the 10-mers, that only 3 of the 4 different types of nucleotides are used. The set is described as "maximal" because the computer programs disclosed in International patent application PCT/US96/09513 provide the largest set for a given input (e.g. length, composition, difference in number of nucleotides between members). Additional minimally cross-hybridizing sets may be formed from subsets of such calculated sets.

When synthesized combinatorially, an oligonucleotide tag of the invention preferably consists of a plurality of subunits, each subunit consisting of an oligonucleotide of 3 to 9 nucleotides in length wherein each subunit is selected from the same minimally cross-hybridizing set. In such embodiments, the number of oligonucleotide tags available depends on the number of subunits per tag and on the length of the subunits.

As used herein in reference to oligonucleotide tags and tag complements, the term "repertoire" means the set of minimally cross-hybridizing set of oligonucleotides that make up the tags in a particular embodiment or the corresponding set of tag complements.

Preferably, in constructing a cDNA library where substantially all different cDNAs have different tags, a tag repertoire is employed whose complexity, or number of distinct tags, greatly exceeds the total number of mRNAs extracted from a cell or tissue sample. Preferably, the complexity of the tag repertoire is at least 10 times that of the polynucleotide population; and more preferably, the complexity of the tag repertoire is at least 100 times that of the polynucleotide population. Below, a protocol is disclosed for cDNA library construction using a primer mixture that contains a full repertoire of exemplary 9-word tags. Such a mixture of tag-containing primers has a complexity of 8^9, or about 1.34×10^8. As indicated by Winslow et al, Nucleic Acids Research, 19: 3251–3253 (1991), mRNA for library construction can be extracted from as few as 10–100 mammalian cells. Since a single mammalian cell contains about 5×10^5 copies of mRNA molecules of about 3.4×10^4 different kinds, by standard techniques one can isolate the mRNA from about 100 cells, or (theoretically) about 5×10^7 mRNA molecules. Comparing this number to the complexity of the primer mixture shows that without any additional steps, and even assuming that mRNAs are converted into cDNAs with perfect efficiency (1% efficiency or less is more accurate), the cDNA library construction protocol results in a population containing no more than 37% of the total number of different tags. That is, without any overt sampling step at all, the protocol inherently generates a sample that comprises 37%, or less, of the tag repertoire. The probability of obtaining a double under these conditions is about 5%, which is within the preferred range. With mRNA from 10 cells, the fraction of the tag repertoire sampled is reduced to only 3.7%, even assuming that all the processing steps take place at 100% efficiency. In fact, the efficiencies of the processing steps for constructing cDNA libraries are very low, a "rule of thumb" being that good library should contain about 10^8 cDNA clones from mRNA extracted from 10^6 mammalian cells.

Use of larger amounts of mRNA in the above protocol, or for larger amounts of polynucleotides in general, where the number of such molecules exceeds the complexity of the tag repertoire, a tag-polynucleotide conjugate mixture potentially contains every possible pairing of tags and types of mRNA or polynucleotide. In such cases, overt sampling may be implemented by removing a sample volume after a serial dilution of the starting mixture of tag-polynucleotide conjugates. The amount of dilution required depends on the amount of starting material and the efficiencies of the processing steps, which are readily estimated.

If mRNA were extracted from 10^6 cells (which would correspond to about 0.5 μg of poly(A)$^+$ RNA), and if primers were present in about 10–100 fold concentration excess—as is called for in a typical protocol, e.g. Sambrook et al, Molecular Cloning, Second Edition, page 8.61 [10 μL 1.8 kb mRNA at 1 mg/mL equals about 1.68×10^{-11} moles and 10

μL 18-mer primer at 1 mg/mL equals about $1.68\times^{-9}$ moles], then the total number of tag-polynucleotide conjugates in a cDNA library would simply be equal to or less than the starting number of mRNAs, or about 5×10^{11} vectors containing tag-polynucleotide conjugates—again this assumes that each step in cDNA construction—first strand synthesis, second strand synthesis, ligation into a vector—occurs with perfect efficiency, which is a very conservative estimate. The actual number is significantly less.

If a sample of n tag-polynucleotide conjugates are randomly drawn from a reaction mixture—as could be effected by taking a sample volume, the probability of drawing conjugates having the same tag is described by the Poisson distribution, $P(r)=e^{-\lambda}(\lambda)^r/r$, where r is the number of conjugates having the same tag and $\lambda=np$, where p is the probability of a given tag being selected. If $n=10^6$ and $p=1/(1.34\times10^8)$, then $\lambda=0.00746$ and $P(2)=2.76\times10^{-5}$. Thus, a sample of one million molecules gives rise to an expected number of doubles well within the preferred range. Such a sample is readily obtained as follows: Assume that the 5×10^{11} mRNAs are perfectly converted into 5×10^{11} vectors with tag-cDNA conjugates as inserts and that the 5×10^{11} vectors are in a reaction solution having a volume of 100 μl. Four 10-fold serial dilutions may be carried out by transferring 10 μl from the original solution into a vessel containing 90 μl of an appropriate buffer, such as TE. This process may be repeated for three additional dilutions to obtain a 100 μl solution containing 5×10^5 vector molecules per μl. A 2 μl aliquot from this solution yields 10^6 vectors containing tag-cDNA conjugates as inserts. This sample is then amplified by straight forward transformation of a competent host cell followed by culturing.

Of course, as mentioned above, no step in the above process proceeds with perfect efficiency. In particular, when vectors are employed to amplify a sample of tag-polynucleotide conjugates, the step of transforming a host is very inefficient. Usually, no more than 1% of the vectors are taken up by the host and replicated. Thus, for such a method of amplification, even fewer dilutions would be required to obtain a sample of 10^6 conjugates.

A repertoire of oligonucleotide tags can be conjugated to a population of polynucleotides in a number of ways, including direct enzymatic ligation, amplification, e.g. via PCR, using primers containing the tag sequences, and the like. The initial ligating step produces a very large population of tag-polynucleotide conjugates such that a single tag is generally attached to many different polynucleotides. However, as noted above, by taking a sufficiently small sample of the conjugates, the probability of obtaining "doubles," i.e. the same tag on two different polynucleotides, can be made negligible. Generally, the larger the sample the greater the probability of obtaining a double. Thus, a design trade-off exists between selecting a large sample of tag-polynucleotide conjugates—which, for example, ensures adequate coverage of a target polynucleotide in a shotgun sequencing operation or adequate representation of a rapidly changing mRNA pool, and selecting a small sample which ensures that a minimal number of doubles will be present. In most embodiments, the presence of doubles merely adds an additional source of noise or, in the case of sequencing, a minor complication in scanning and signal processing, as microparticles giving multiple fluorescent signals can simply be ignored.

As used herein, the term "substantially all" in reference to attaching tags to molecules, especially polynucleotides, is meant to reflect the statistical nature of the sampling procedure employed to obtain a population of tag-molecule conjugates essentially free of doubles. The meaning of substantially all in terms of actual percentages of tag-molecule conjugates depends on how the tags are being employed. Preferably, for nucleic acid sequencing, substantially all means that at least eighty percent of the polynucleotides have unique tags attached. More preferably, it means that at least ninety percent of the polynucleotides have unique tags attached. Still more preferably, it means that at least ninety-five percent of the polynucleotides have unique tags attached. And, most preferably, it means that at least ninety-nine percent of the polynucleotides have unique tags attached.

Tags can be conjugated to cDNAs of existing libraries by standard cloning methods. cDNAs are excised from their existing vector, isolated, and then ligated into a vector containing a repertoire of tags. Preferably, the tag-containing vector is linearized by cleaving with two restriction enzymes so that the excised cDNAs can be ligated in a predetermined orientation. The concentration of the linearized tag-containing vector is in substantial excess over that of the cDNA inserts so that ligation provides an inherent sampling of tags.

A general method for exposing the single stranded tag after amplification involves digesting a target polynucleotide-containing conjugate with the 5'→3' exonuclease activity of T4 DNA polymerase, or a like enzyme, e.g. as described in Kuijper et al, Gene, 112: 147–155 (1992). When used in the presence of a single deoxynucleoside triphosphate, such a polymerase will cleave nucleotides from 3' recessed ends present on the non-template strand of a double stranded fragment until a complement of the single deoxynucleoside triphosphate is reached on the template strand. When such a nucleotide is reached the 5'→3' digestion effectively ceases, as the polymerase's extension activity adds nucleotides at a higher rate than the excision activity removes nucleotides. Consequently, single stranded tags constructed with three nucleotides are readily prepared for loading onto solid phase supports.

After the oligonucleotide tags are prepared for specific hybridization, e.g. by rendering them single stranded as described above, the polynucleotides are mixed with microparticles containing the complementary sequences of the tags under conditions that favor the formation of perfectly matched duplexes between the tags and their complements. There is extensive guidance in the literature for creating these conditions. Exemplary references providing such guidance include Wetmur, Critical Reviews in Biochemistry and Molecular Biology, 26: 227–259 (1991); Sambrook et al, Molecular Cloning: A Laboratory Manual, 2nd Edition (Cold Spring Harbor Laboratory, New York, 1989); and the like. Preferably, the hybridization conditions are sufficiently stringent so that only perfectly matched sequences form stable duplexes. Under such conditions the polynucleotides specifically hybridized through their tags may be ligated to the complementary sequences attached to the microparticles. Finally, the microparticles are washed to remove polynucleotides with unligated and/or mismatched tags.

Preferably, for sequencing applications, standard CPG beads of diameter in the range of 20–50 μm are loaded with about 10^5 polynucleotides, and glycidalmethacrylate (GMA) beads available from Bangs Laboratories (Carmel, Ind.) of diameter in the range of 5–10 μm are loaded with a few tens of thousand polynucleotide, e.g. 4×10^4 to 6×10^4, to a hundred thousand polynucleotides.

DNA Sequencing

Polynucleotides loaded onto microparticles may be simultaneously sequenced in the instant apparatus using a "base-by-base" DNA sequencing methodology. Such sequencing

methodology permits the stepwise identification of a sequence of nucleotides in a target polynucleotide, usually one base at a time, through successive cycles of treatment and detection. Base-by-base approaches are disclosed in the following references: Cheeseman, U.S. Pat. No. 5,302.509; Tsien et al, International application WO 91/06678; Rosenthal et al, International application WO 93/21340; Canard et al, Gene, 148: 1–6 (1994); Metzker et al, Nucleic Acids Research, 22: 4259–4267 (1994); and the like. Preferably, the base-by-base approach disclosed by Brenner in U.S. Pat. No. 5,599,675 is used with the apparatus of the invention to sequence polynucleotides on a population of loaded microparticles disposed as a planar array in the flow chamber. Accordingly, Brenner, U.S. Pat. No. 5,599,675 is incorporated by reference. Preferably, the a population of loaded microparticles for sequencing includes at least ten thousand loaded microparticles; more preferably, such a population includes at least fifty thousand loaded microparticles; and still more preferably, such a population includes at least one hundred thousand loaded microparticles.

Preferably, the sequencing method of Brenner (cited above) is employed in the embodiment disclosed in Albrecht et al International patent application PCT/US97/09472 which discloses the use of encoded adaptors. An encoded adaptor is a doable stranded oligonucleotide comprising a protruding strand and an oligonucleotide tag selected from a minimally cross-hybridizing set of oligonucleotides. Encoded adaptors whose protruding strands form perfectly matched duplexes with the complementary protruding strands of the target polynucleotide are ligated. After ligation, the identity and ordering of the nucleotides in the protruding strands are determined, or "decoded," by specifically hybridizing a labeled tag complement to its corresponding tag on the ligated adaptor. Encoded adaptors may be used in an adaptor-based method of DNA sequencing that includes repeated cycles of ligation, identification, and cleavage, such as the method described in Brenner (cited above). Briefly, such a method comprises the following steps: (a) ligating an encoded adaptor to an end of a polynucleotide, the encoded adaptor having a nuclease recognition site of a nuclease whose cleavage site is separate from its recognition site; (b) identifying one or more nucleotides at the end of the polynucleotide by the identity of the encoded adaptor ligated thereto; (c) cleaving the polynucleotide with a nuclease recognizing the nuclease recognition site of the encoded adaptor such that the polynucleotide is shortened by one or more nucleotides; and (d) repeating said steps (a) through (c) until said nucleotide sequence of the polynucleotide is determined. In the identification step, successive sets of tag complements are specifically hybridized to the respective tags carried by encoded adaptors ligated to the ends of the target polynucleotides, as described above. The type and sequence of nucleotides in the protruding strands of the polynucleotides are identified by the label carried by the specifically hybridized tag complement and the set from which the tag complement came.

Construction and Sorting of cDNA Library for Signature Sequencing with Encoded Adaptors

In this example, a cDNA library is constructed in which an oligonucleotide tag consisting of 8 four-nucleotide "words" is attached to each cDNA. As described above, the repertoire of oligonucleotide tags of this size is sufficiently large (about 10^8) so that if the cDNAs are synthesized from a population of about 10^6 mRNAs, then there is a high probability that each cDNA will have a unique tag for sorting. After mRNA extraction, first strand synthesis is carried out in the presence of 5-Me-dCTP (to block certain cDNA restriction sites) and a biotinylated primer mixture containing the oligonucleotide tags. After conventional second strand synthesis, the tag-cDNA conjugates are cleaved with Dpn II (which is unaffected by the 5-Me-deoxycytosines), the biotinylated portions are separated from the reaction mixture using streptavidin-coated magnetic beads, and the tag-cDNA conjugates are recovered by cleaving them from the magnetic beads via a Bsm BI site carried by the biotinylated primer. The Bsm BI-Dpn II fragment containing the tag-cDNA conjugate is then inserted into a plasmid and amplified. After isolation of the plasmids, tag-cDNA conjugates are amplified out of the plasmids by PCR in the presence of 5-Me-dCTP, using biotinylated and fluorescently labeled primers containing pre-defined restriction endonuclease sites. After affinity purification with streptavidin coated magnetic beads, the tag-cDNA conjugates are cleaved from the beads, treated with T4 DNA polymerase in the presence of dGTP to render the tags single stranded, and then combined with a repertoire of GMA beads having tag complements attached. After stringent hybridization and ligation, the GMA beads are sorted via FACS to produce an enriched population of GMA beads loaded with cDNAs. The enriched population of loaded GMA beads are immobilized in a planar array in a flow chamber where base-by-base sequence takes place using encoded adaptors, as disclosed in Albrecht et al, International patent application PCT/US97/09472.

Approximately 5 μg of poly(A^+) mRNA is extracted from DBY746 yeast cells using conventional protocols. First and second strand cDNA synthesis is carried out by combining 100–150 pmoles of the following primer (SEQ ID NO: 1):

5'-biotin-ACTAATCGTCTCACTATTTAATTAA[W,W,W,G]$_8$CC(T)$_{18}$V-3'

with the poly(A+) mRNA using a Stratagene (La Jolla, Calif.) cDNA Synthesis Kit in accordance with the manufacturer's protocol. This results in cDNAs whose first stand deoxycytosines are methylated at the 5-carbon position. In the above formula, "V" is G, C, or A, "[W, W, W, G]" is a four-nucleotide word selected from Table II of Brenner, International patent application PCT/US96/09513, the single underlined portion is a Bsm BI recognition site, and the double underlined portion is a Pac I recognition site. After size fractionation (GIBCO-BRL cDNA Size Fractionation Kit) using conventional protocols, the cDNAs are digested with Dpn II (New England Bioscience, Beverly, Mass.) using manufacturer's protocol and affinity purified with streptavidin-coated magnetic beads (M-280 beads, Dynal A. S., Oslo, Norway). The DNA captured by the beads is digested with Bsm BI to release the tag-cDNA conjugates for cloning into a modified pBCSK$^-$ vector (Stratagene, La Jolla, Calif.) using standard protocols. The pBCSK$^-$ vector is modified by adding a Bbs I site by inserting the following fragment (SEQ ID NO: 2) into the Kpn I/Eco RV digested vector.

CGAAGACCC
3'-CATGGCTTCTGGGGATA-5'

Bsm BI/Dpn II digested tag-cDNA conjugate is inserted in the pBCSK$^-$ which is previously digested with Bbs I and Bam HI. After ligation, the vector is transfected into the manufacturer's recommended host for amplification.

After isolating the above pBCSK⁻ vector from a standard plasmid miniprep, the tag-cDNA conjugates are amplified by PCR in the presence of 5-Me-dCTP using 20-mer primers complementary to vector sequences flanking the tag-cDNA insert. The "upstream" primer, i.e. adjacent to the tag, is biotinylated and the "downstream" primer, i.e. adjacent to the cDNA, is labeled with fluorescein. After amplification, the PCR product is affinity purified then cleaved with Pac I to release fluorescently labeled tag-cDNA conjugates. The tags of the conjugates are rendered single stranded by treating them with T4 DNA polymerase in the presence of dGTP. After the reaction is quenched, the tag-cDNA conjugate is purified by phenol-chloroform extraction and combined with 5.5 mm GMA beads carrying tag complements, each tag complement having a 5' phosphate. Hybridization is conducted under stringent conditions in the presence of a thermal stable ligase so that only tags forming perfectly matched duplexes with their complements are ligated. The GMA beads are washed and the loaded beads are concentrated by FACS sorting, using the fluorescently labeled cDNAs to identify loaded GMA beads. The tag-cDNA conjugates attached to the GMA beads are digested with Dpn II to remove the fluorescent label and treated with alkaline phosphatase to prepare the cDNAs for sequencing. That is, phasphatase is used to remove the 5' phosphate from the ends of the cDNAs to prevent unwanted cDNA-cDNA ligations by way of the palindromic Dpn II site.

The following cleavage adaptor (SEQ ID NO:3) is ligated to the Dpn II-digested and phosphatase treated cDNAs:

```
5'-pGATCAGCTGCTGCAAATTT
      pTCGACGACGTTTAAA
```

After ligation, the 3' phosphate is removed by alkaline phosphatase, the 5' strand of the cDNA is treated with T4 DNA kinase, and the nick between the cleavage adaptor and cDNA is ligated. After cleavage by Bbv I, encoded adaptors are ligated to the ends of the cDNAs and the beads are ready for loading into the flow chamber.

Ligation of the adaptors to the target polynucleotide is carried out in a mixture consisting of 5 μl beads (20 mg), 3 μL NEB 10×ligase buffer, 5 μL adaptor mix (25 nM), 2.5 μL NEB T4 DNA ligase (2000 units/μL), and 14.5 μL distilled water. The mixture is incubated at 16° C. for 30 minutes, after which the beads are washed 3 times in TE (pH 8.0).

After centrifugation and removal of TE, the 3' phosphates of the ligated adaptors are removed by treating the polynucleotide-bead mixture with calf intestinal alkaline phosphatase (CIP) (New England Biolabs, Beverly, Mass.), using the manufacturers protocol. After removal of the 3' phosphates, the CIP may be inactivated by proteolytic digestion, e.g. using Pronase™ (available form Boeringer Mannhiem, Indianapolis, Ind.), or an equivalent protease, with the manufacturer's protocol. The polynucleotide-bead mixture is then washed, treated with a mixture of T4 polynucleotide kinase and T4 DNA ligase (New England Biolibs, Beverly, Mass.) to add a 5' phosphate at the gap between the target polynucleotide and the adaptor, and to complete the ligation of the adaptors to the target polynucleotide. The bead-polynucleotide mixture is then wased in TE, diluted to a concentration of approximately 100 thousand beads per μL, and 5 μL of the resulting solution is loaded into a flow chamber with the help of the holders of FIG. **4**.

The top strands of the following 16 sets of 64 encoded adaptors (SEQ ID NO: 4 through SEQ ID NO: 19) are each separately synthesized on an automated DNA synthesizer (model 392 Applied Biosystems, Foster City) using standard methods. The bottom strand, (SEQ ID NO: 20), which is the same for all adaptors, is synthesized separately then hybridized to the respective top strands:

SEQ ID NO.	Encoded Adaptor
4	5'-pANNNTACAGCTGCATCCCttggcgctgagg pATGCACGCGTAGGG-5'
5	5'-pNANNTACAGCTGCATCCCtgggcctgtaag pATGCACGCGTAGGG-5'
6	5'-pCNNNTACAGCTGCATCCCttgacgggtctc pATGCACGCGTAGGG-5'
7	5'-pNCNNTACAGCTGCATCCCtgcccgcacagt pATGCACGCGTAGGG-5'
8	5'-pGNNNTACAGCTGCATCCCttcgcctcggac pATGCACGCGTAGGG-5'
9	5'-pNGNNTACAGCTGCATCCCtgatccgctagc pATGCACGCGTAGGG-5'
10	5'-pTNNNTACAGCTGCATCCCttccgaacccgc pATGCACGCGTAGGG-5'
11	5'-pNTNNTACAGCTGCATCCCtgaggggggatag pATGCACGCGTAGGG-5'
12	5'-pNNANTACAGCTGCATCCCttcccgctacac pATGCACGCGTAGGG-5'
13	5'-pNNNATACAGCTGCATCCCtgactccccgag pATGCACGCGTAGGG-5'
14	5'-pNNCNTACAGCTGCATCCCtgtgttgcgcgg pATGCACGCGTAGGG-5'
15	5'-pNNNCTACAGCTGCATCCCtctacagcagcg pATGCACGCGTAGGG-5'
16	5'-pNNGNTACAGCTGCATCCCtgtcgcgtcgtt pATGCACGCGTAGGG-5'
17	5'-pNNNGTACAGCTGCATCCCtcggagcaacct pATGCACGCGTAGGG-5'
18	5'-pNNTNTACAGCTGCATCCCtggtgaccgtag pATGCACGCGTAGGG-5'
19	5'-pNNNTTACAGCTGCATCCCtcccctgtcgga pATGCACGCGTAGGG-5'

where N and p are as defined above, and the nucleotides indicated in lower case letters are the 12-mer oligonucleotide tags. Each tag differs from every other by 6 nucleotides. Equal molar quantities of each adaptor are combined in NEB #2 restriction buffer (New England Biolabs, Beverly, Mass.) to form a mixture at a concentration of 1000 pmol/μL.

Each of the 16 tag complements are separately synthesized as amino-derivatized oligonucleotides and are each labeled with a fluorescein molecule (using an NHS-ester of fluorescein, available from Molecular Probes, Eugene, Oreg.) which is attached to the 5' end of the tag complement through a polyethylene glycol linker (Clonetech Laboratories, Palo Alto, Calif.). The sequences of the tag complements are simply the 12-mer complements of the tags listed above.

A flow chamber of the design shown in FIGS. **2***a* and **2***b* is employed in association with an Olympus Optical Co., Ltd. (Tokyo, Japan) model BX60MF5 fluorescent microscope fitted with a model U-ULS75XE 75 watt Xenon arc lamp, a motorized filter wheel, a Ludl Electronic Products, Ltd. computer-controlled stage, and a Photometrics, Ltd. (Tucson, Ariz.) PXL CCD camera with a 2000×2000 pixel array. Appropriate bandpass filters (**122**) and (**124**) are

employed for exciting fluorescein and transmitting fluorescent signal to CCD camera (**120**). Microparticle positions are determined by top-lighting with broadband light from Xenon lamp (**126**) reduced by a factor of about 10^{-4} with a neutral density filter. Fluorescent images are collected with about 2 minute exposure times.

Height (**204**) of flow chamber (**201**) is selected to be 7 μm, or approximately 140% of the diameter of the GMA beads. Width (**210**) of flow chamber (**201**) is selected so as to ensure that a 3×3 array of 9 image pixels will cover approximately 40–60% of a bead's image after 10×magnification (as illustrated in FIG. **7**). Thus, in order to capture images of tiles of about 100 thousand 5 μm GMA beads, width (**210**) is selected to have a value of 1.7 mm. Length (**212**) is selected so that the flow chamber can hold from 1 to 10 tiles of about one hundred thousand 5 μm diameter beads each. The cross section (**220**) of inlet passage (**214**) matches that of the inlet tubing and gradually enlarges to match that of flow chamber (**201**) in the region of the planar cavity, i.e. the region holding the GMA beads on which analysis is performed. It is desirable to have a constant cross section through the planar cavity of flow chamber (**201**) to minimize the creation of non-uniform flow patterns, as might occur with sudden constrictions and/or expansions in cross section. Both body (**218**) and cover (**216**) of flow chamber (**201**) are glass, and the planar cavity and channels of body (**218**) are formed by standard chemical etching techniques. Cross section (**222**) of outlet passage (**224**) is selected to match the cross section of flow chamber (**201**) at dam (**202**).

The fluidics system of FIG. **5***a* which includes all valves, syringe pump (**500**), and Peltier block (**152**), is controlled by code written in LabVIEW 5.0 (National Instruments, Austin, Tex.) and run on a Compact Deskpro Pentium-based microprocessor, which is connected to the various components of the fluidics system by standard I/O circuit boards. Detection system (**114**) and overall control of the instrument is effected through a Sun Microsystems (Mountain View, Calif.) Sparcstation 5.

Three cycles of ligation, identification, and cleavage are carried out in flow chamber (**201**) to give the sequences of 12 nucleotides at the termini of each of approximately 500,000 cDNAs. That is, five tiles of GMA beads are analyzed in the following series of process steps:

1. Calibrate focal plane of GMA beads.
2. Hybridize decoder.
3. Autofocus on tile 1.
4. Set focus to bead centers.
5. Collect fluorescent image.
6. Set focus to bead focal plane (scatter centers).
7. Collect image.
8. Repeat steps 4–7 for remaining tiles.
9. Wash.
10. Repeat steps 2–9 for remaining decoders.
11. Cleave encoded adaptor.
12. Wash.
13. Ligate top strand of next encoded adaptor.
14. Wash.
15. Repeat steps 13–14.
16. Kinase bottom strand of encoded adaptor.
17. Wash.
18. Ligate bottom strand of encoded adaptor.
19. Wash.
20. Repeat steps 2–9.
21. Repeat steps 11–19 for next encoded adaptor.

In steps 2–9, nucleotides of the cDNAs are identified by hybridizing tag complements to the encoded adaptors. Specifically hybridized tag complements are detected by exciting their fluorescent labels with illumination beam (**110**) from Xenon arc lamp (**126**). In step 13, encoded adaptors and T4 DNA ligase (Promega, Madison, Wis.) at about 0.75 units per μL are passed through the flow chamber at a flow rate of about 1–2 μL per minute for about 20–30 minutes at 16° C., after which wash of step 14 is executed by flowing, in succession, a solution of Pronase™ (Boehringer Mannheim, Indianapolis, Ind.), a salt wash solution, and an ethanol wash solution through the flow chamber, all with the same flow rate of 1–2 μL per minute and for durations of 15, 10, and 10 minutes, respectively. The salt wash solution is 150 mM NaCl and 10 mM Tris-HCl (pH 8.5), and the ethanol wash solution is 3:1 (v/v) solution of the salt wash solution and ethanol. The ligation and wash steps 13 and 14 are repeated once, after which the adaptors and the cDNAs are prepared for second strand ligation by passing T4 DNA kinase (New England Bioscience, Beverly, Mass.) at 7 units per μL through the flow cham ber at 37° C. with a flow rate of 1–2 μL per minute for 15–20 minutes. Ligation of the second strand is carried out by flowing T4 DNA ligase (0.75 units per mL, Promega) through the flow chamber for 20–30 minutes at a rate of 1–2 μL per minute, followed by Pronase™ treatment and washing as described above. Tag complements at 25 nM concentration are passed through the flow chamber at a flow rate of 1–2 μL per minute for 10 minutes at 20° C., after which the fluorescent labels carried by the tag complements are illuminated and fluorescence is collected. The tag complements are melted from the encoded adaptors by passing NEB #2 restriction buffer with 3 mM $MgCl_2$ through the flow chamber at a flow rate of 1–2 μL per minute at 55° C. for 10 minutes. Encoded adaptors are cleaved from the cDNAs by passing Bbv I (New England Biosciences, Beverly, Mass.) at 1 unit/μL at a flow rate of 1–2 μL per minute for 20 minutes at 37° C., followed by Pronase™ treatment and washing, as described above.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 20

<210> SEQ ID NO 1
<211> LENGTH: 78
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer
<221> NAME/KEY: misc_feature
<222> LOCATION: (26)...(57)
<223> OTHER INFORMATION: n = A,T,C or G

-continued

<400> SEQUENCE: 1

actaatcgtc tcactattta attaannnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnggt 60

ttttttttttt tttttttv 78

<210> SEQ ID NO 2
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic fragment

<400> SEQUENCE: 2

ataggggtct tcggtac 17

<210> SEQ ID NO 3
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic cleavage adaptor

<400> SEQUENCE: 3

gatcagctgc tgcaaattt 19

<210> SEQ ID NO 4
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 4

annntacagc tgcatccctt ggcgctgagg 30

<210> SEQ ID NO 5
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 5

nanntacagc tgcatccctg ggcctgtaag 30

<210> SEQ ID NO 6
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 6

cnnntacagc tgcatccctt gacgggtctc 30

<210> SEQ ID NO 7
<211> LENGTH: 30

-continued

<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 7

ncnntacagc tgcatccctg cccgcacagt 30

<210> SEQ ID NO 8
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 8

gnnntacagc tgcatccctt cgcctcggac 30

<210> SEQ ID NO 9
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 9

ngnntacagc tgcatccctg atccgctagc 30

<210> SEQ ID NO 10
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 10

tnnntacagc tgcatccctt ccgaacccgc 30

<210> SEQ ID NO 11
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 11

ntnntacagc tgcatccctg agggggatag 30

<210> SEQ ID NO 12
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor

-continued

<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 12

nnantacagc tgcatccctt cccgctacac 30

<210> SEQ ID NO 13
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(3)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 13

nnnatacagc tgcatccctg actccccgag 30

<210> SEQ ID NO 14
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 14

nncntacagc tgcatccctg tgttgcgcgg 30

<210> SEQ ID NO 15
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(3)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 15

nnnctacagc tgcatccctc tacagcagcg 30

<210> SEQ ID NO 16
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 16

nngntacagc tgcatccctg tcgcgtcgtt 30

<210> SEQ ID NO 17
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(3)
<223> OTHER INFORMATION: n = A,T,C or G

-continued

<400> SEQUENCE: 17

nnngtacagc tgcatccctc ggagcaacct 30

<210> SEQ ID NO 18
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 18

nntntacagc tgcatccctg gtgaccgtag 30

<210> SEQ ID NO 19
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic encoded adaptor
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(3)
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 19

nnnttacagc tgcatccctc ccctgtcgga 30

<210> SEQ ID NO 20
<211> LENGTH: 14
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic oligonucleotide

<400> SEQUENCE: 20

gggatgcgca cgta 14

We claim:

1. A device-readable medium embodying a program of instructions for execution by said device to perform a method of generating images of a planar array of microparticles and tracking positions of the microparticles during a sequence of processing steps, said program of instructions comprising instructions for:

rendering a plurality of digital images of the planar array of the microparticles during the sequence of processing steps, based on optical signals generated at the microparticles; and

processing the plurality of digital images, wherein said processing includes correlating the optical signals generated at each microparticle with its corresponding image in each of the plurality of digital images.

2. The device-readable medium of claim **1**, wherein said processing further includes determining the approximate center of each microparticle, based on a recorded optical characteristic of said microparticle.

3. The device-readable medium of claim **2**, wherein said processing further includes assigning a plurality of pixels to each microparticle, for determining at least one property of the optical signals generated at each microparticle.

4. The device-readable medium of claim **3**, wherein the number of pixels assigned to a given microparticle is determined based on at least one of:

(i) the degree to which the approximated center of the given microparticle is likely to deviate from the geometric center;

(ii) the size of the given microparticle; and

(iii) the uniformity of the diameter and shape of the given microparticle.

5. The device-readable medium of claim **3**, wherein an initial pixel of the plurality of pixels is assigned to each microparticle which encloses the approximated center of that microparticle.

6. The device-readable medium of claim **5**, wherein said processing further includes assigning additional pixels, immediately adjacent the initial pixel, to each microparticle, after the initial pixel is assigned.

* * * * *

EXHIBIT 6

US007232656B2

(12) **United States Patent**
Balasubramanian et al.

(10) **Patent No.: US 7,232,656 B2**
(45) **Date of Patent: *Jun. 19, 2007**

(54) **ARRAYED BIOMOLECULES AND THEIR USE IN SEQUENCING**

(75) Inventors: **Shankar Balasubramanian**, Waldon (GB); **David Klenerman**, Waldon (GB); **Colin Barnes**, Waldon (GB); **Mark Allen Osborne**, Waldon (GB)

(73) Assignee: **Solexa Ltd.**, Essex (GB)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 427 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **10/610,305**

(22) Filed: **Jun. 30, 2003**

(65) **Prior Publication Data**

US 2004/0091903 A1 May 13, 2004

Related U.S. Application Data

(63) Continuation of application No. 09/771,708, filed on Jan. 30, 2001, now Pat. No. 6,787,308, which is a continuation-in-part of application No. PCT/GB99/02487, filed on Jul. 30, 1999.

(30) **Foreign Application Priority Data**

Jul. 30, 1998	(EP)	98306094
Oct. 16, 1998	(GB)	9822670.7
Feb. 1, 2000	(GB)	0002310.1

(51) **Int. Cl.**
C12Q 1/68 (2006.01)
C07H 21/02 (2006.01)
C07H 21/04 (2006.01)

(52) **U.S. Cl.** **435/6**; 536/23.1; 536/24.3

(58) **Field of Classification Search** 435/6; 536/23.1, 24.3

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,302,509	A		4/1994	Cheeseman
5,314,829	A		5/1994	Coles
5,634,413	A		6/1997	Listner et al.
5,643,768	A		7/1997	Kawasaki
5,658,736	A	*	8/1997	Wong 435/6
5,658,754	A		8/1997	Kawasaki
5,709,999	A	*	1/1998	Shattuck-Eidens et al. 435/6
5,780,231	A		7/1998	Brenner

FOREIGN PATENT DOCUMENTS

DE	196 12 356 A1		10/1997
EP	0 665 293 A2		8/1995
EP	0 721 016 A2		7/1996
EP	0 853 129 A2		7/1998
EP	0 995 804 A2		4/2000
WO	WO 89/03432		4/1989
WO	WO 90/13666		11/1990
WO	WO 93/21340		10/1993
WO	WO 95/20053		7/1995
WO	WO 96/27025	*	9/1996
WO	WO 96/36731		11/1996
WO	WO 97/04131		2/1997
WO	WO 97/08183		3/1997
WO	WO 98/03673		1/1998
WO	WO 98/20019		5/1998
WO	WO 98/29376		7/1998
WO	WO 99/05315		2/1999
WO	WO 99/05321		2/1999
WO	WO 99/28494		6/1999
WO	WO 99/28505		6/1999

OTHER PUBLICATIONS

Pastinen et al., "Minisequencing: A Specific Tool for DNA Analysis and Diagnostics on Oligonucleotide Arrays," Genome Research, Jun. 1997, vol. 7, No. 6, pp. 606-614).*

Ronaghi et al., "PCR-Introduced Loop Structure as Primer in DNA Sequencing," Biotechniques, Nov. 1998, vol. 25, pp. 876-884.*

2003 Release: International Consortium Completes HGP; International Consortium Completes Human Genome Project; pp. 1-7; Apr. 14, 2003.

CNN.com; Genome announcement a milestone, but only a beginning; pp. 1-6; Jun. 26, 2000.

S. Foder et al., "Combinatorial Chemistry—Applications of Light-Directed Chemical Synthesis", *Trends in Biotechnology*, Jan. 1994, vol. 12, pp. 19-26.

M. Schena et al., "Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarry", *SCIENCE*, Oct. 20, 1995, vol. 270, pp. 467-470.

S. Tyagi et al., "Molecular Beacons: Probes that Fluoresce Upon Hybridization", *Nature Biotechnology*, Mar. 1996, vol. 14, pp. 303-308.

M. Bulyk et al., "Quantifying DNA-Protein Interactions by Double-Stranded DNA Arrays", *Nature Biotechnology*, Jun. 1999, vol. 17, pp. 573-577.

R. Vale et al., "Direct Observation of Single Kinesin Molecules Moving Along Microtubules", *NATURE*, Apr. 1996, vol. 380, pp. 451-453.

P. Moyer et al., "Near-Field Optical Microscopes Break the Diffraction Limit", *Laser Focus World*, Oct. 1993, pp. 105-109.

H. Hansma et al., "Biomolecular Imaging with the Atomic Force Microscope", *Annu. Rev. Biophys. Biomol. Struct.*, 1994, vol. 23, pp. 115-139.

G. Binnig et al., "Scanning Tunneling Microscopy", *Helvetica Physica Acta*. vol. 55, 1982, pp. 726-735.

A. Mirzabekov, "DNA Sequencing by Hybridization—A Megasequencing Method and a Diagnostic Tool?", *Trends in Biotechnology*, Jan. 1994, vol. 12, pp. 27-32.

* cited by examiner

Primary Examiner—Ethan Whisenant
(74) *Attorney, Agent, or Firm*—Klauber & Jackson

(57) **ABSTRACT**

The invention is directed to a method for analysing genome wide variation in an individual. The method comprises randomly fragmenting the individual's genome and generating sequence reads of multiple bases on all fragments of the individual's genome, aligning the sequence reads generated with a known genomic reference sequence, and analysing variations between the sequence reads derived from the individual's genome and the known genomic reference sequence.

9 Claims, 5 Drawing Sheets

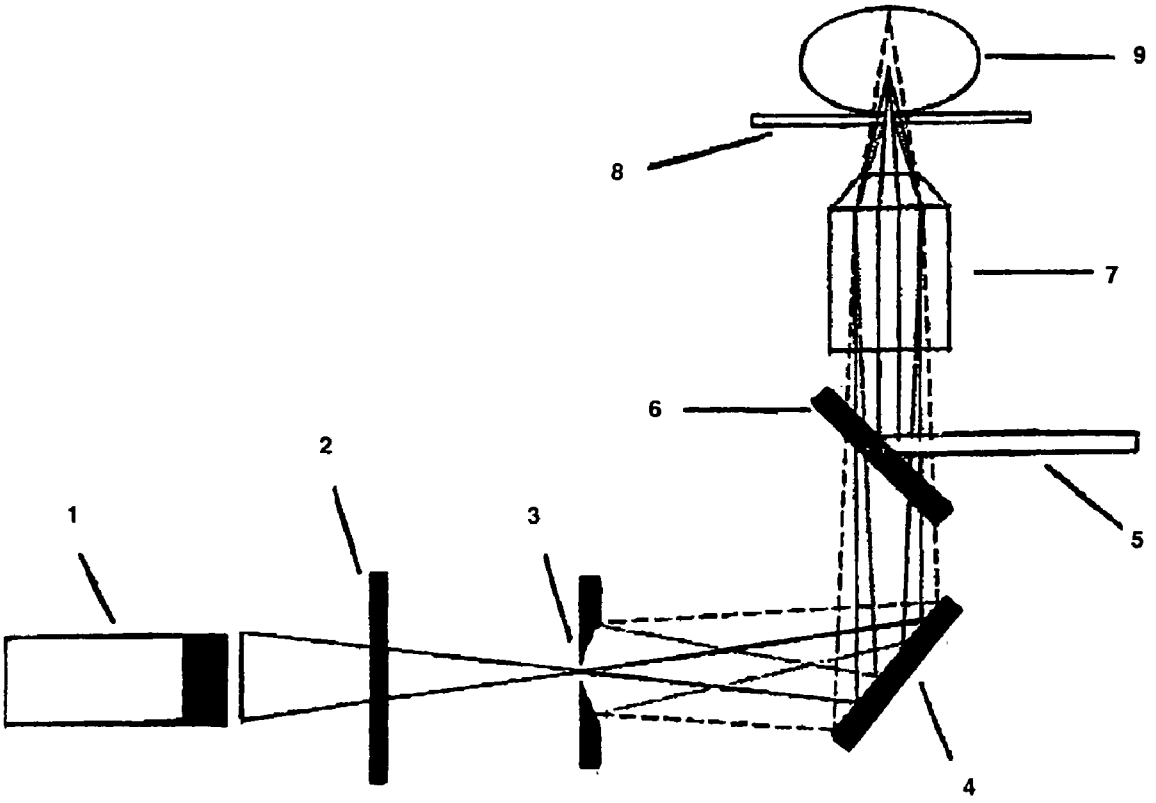


FIG. 1

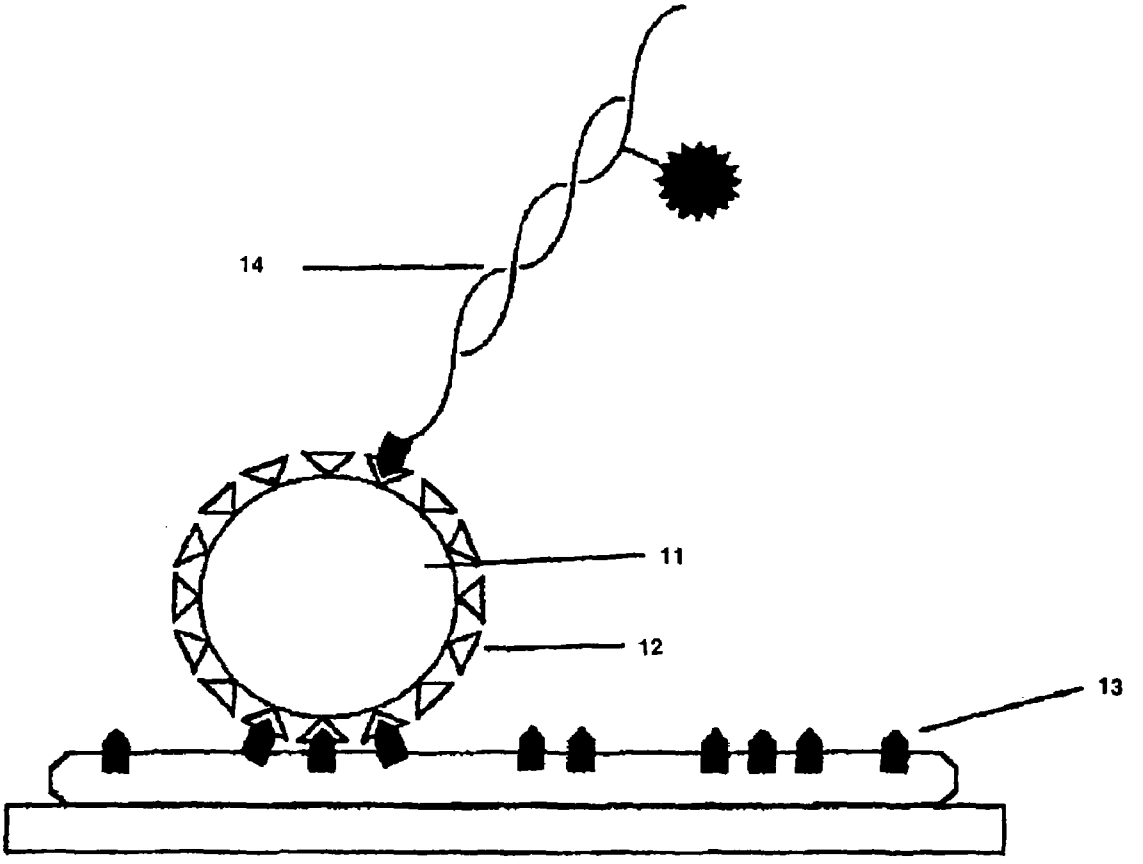


FIG. 2

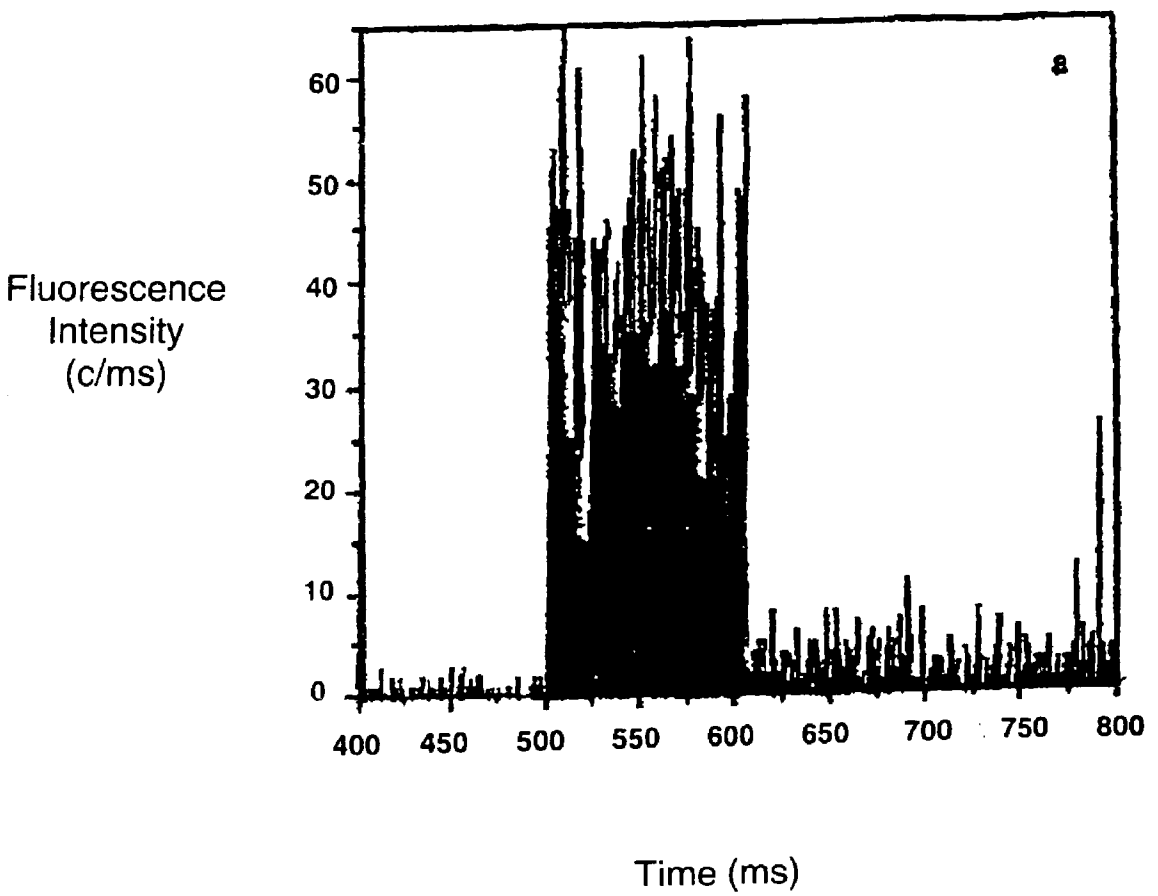

Fluorescence Intensity (c/ms)
60
50
40
30
20
10
0
400
450
500
550
600
650
700
750
800
Time (ms)

FIG. 3

FIG. 4A

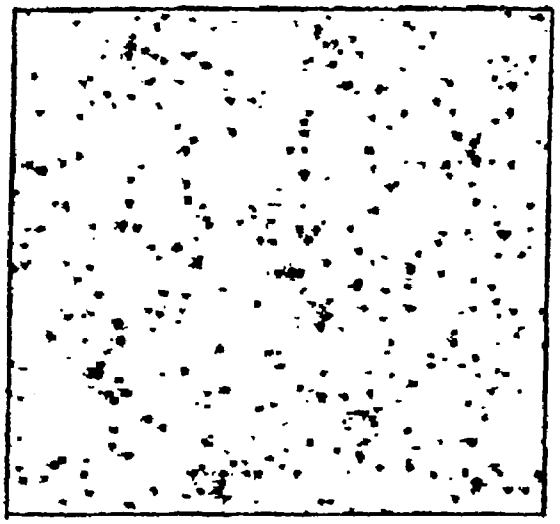


FIG. 4B

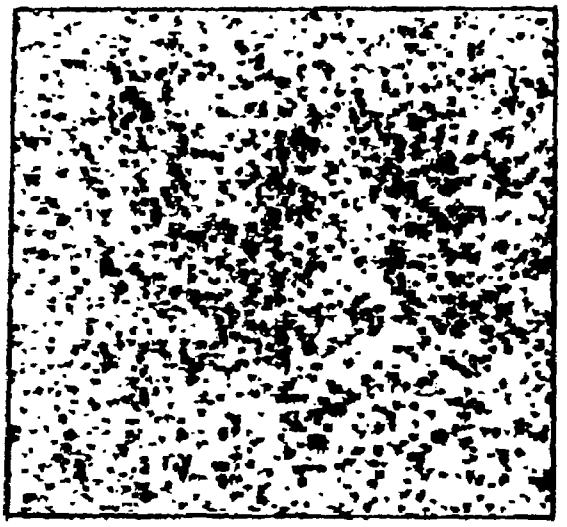




FIG. 4C

FIG. 5A

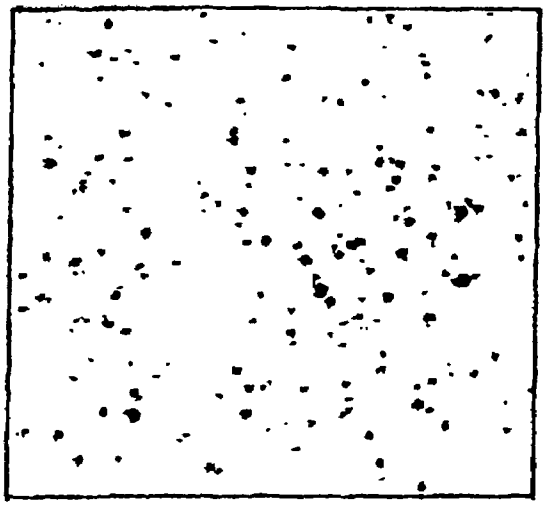


FIG. 5B

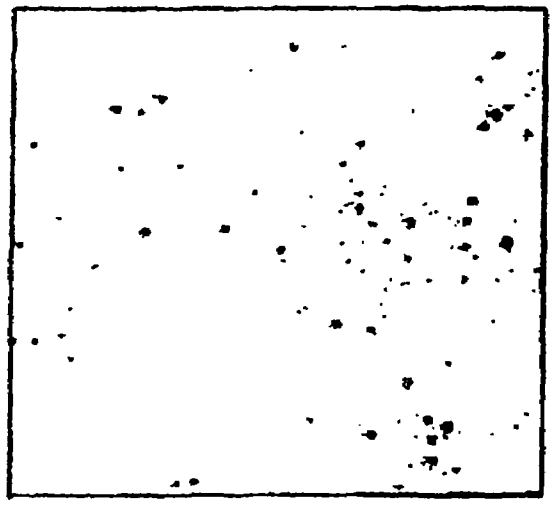
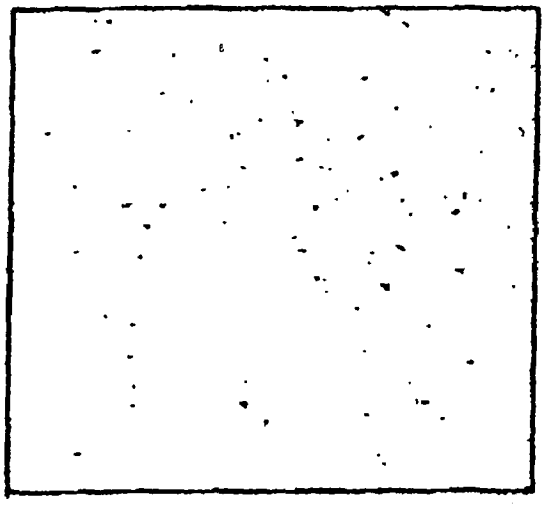
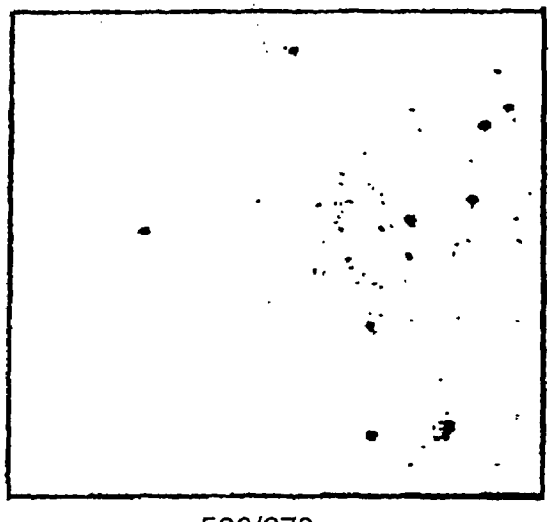


FIG. 5C



532/670 nm

FIG. 5D



580/670 nm

ARRAYED BIOMOLECULES AND THEIR USE IN SEQUENCING

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a Continuation of application Ser. No. 09/771,708 filed Jan. 30, 2001, now U.S. Pat. No. 6,787,308, issued Sep. 7, 2004, which is a continuation in part of International Application No. PCT/GB99/02487, which designated the United States and was filed on Jul. 30, 1999, was published in English, and which claims the benefit of British Application GB9822670.7, filed Oct. 16, 1998, and also claims benefit of European Application EP98306094.8, filed Jun. 30, 1998. The entire teachings of the above applications are incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to fabricated arrays of molecules, and to their analytical applications. In particular, this invention relates to the use of fabricated arrays in methods for obtaining genetic sequence information.

BACKGROUND OF THE INVENTION

Advances in the study of molecules have been led, in part, by improvement in technologies used to characterise the molecules or their biological reactions. In particular, the study of nucleic acids, DNA and RNA, has benefited from developing technologies used for sequence analysis and the study of hybridisation events.

An example of the technologies that have improved the study of nucleic acids, is the development of fabricated arrays of immobilised nucleic acids. These arrays typically consist of a high-density matrix of polynucleotides immobilized onto a solid support material Fodor et al., Trends in Biotechnology (1994) 12.19–26, describes ways of assembling the nucleic acid arrays using a chemically sensitised glass surface protected by a mask, but exposed at defined areas to allow attachment of suitably modified nucleotides. Typically, these arrays may be described as "many molecule" arrays, as distinct regions are formed on the solid support comprising a high density of one specific type of polynucleotide.

An alternative approach is described by Schena et al., Science (1995) 270.467–470, where samples of DNA are positioned at predetermined sites on a glass microscope slide by robotic micropipetting techniques. The DNA is attached to the glass surface along its entire length by non-covalent electrostatic interactions. However, although hybridisation with complementary DNA sequences can occur, this approach may not permit the DNA to be freely available for interacting with other components such as polymerase enzymes, DNA-binding proteins etc.

Recently, the Human Genome Project determined the entire sequence of the human genome-all 3×10^9 bases. The sequence information represents that of an average human. However, there is still considerable interest in identifying differences in the genetic sequence between different individuals. The most common form of genetic variation is single nucleotide polymorphisms (SNPs). On average one base in 1000 is a SNP, which means that there are 3 million SNPs for any individual. Some of the SNPs are in coding regions and produce proteins with different binding affinities or properties. Some are in regulatory regions and result in a different response to changes in levels of metabolites or

messengers. SNPs are also found in noncoding regions, and these are also important as they may correlate with SNPs in coding or regulatory regions. The key problem is to develop a low cost way of determining one or more of the SNPs for an individual.

The nucleic acid arrays may be used to determine SNPs, and they have been used to study hybridisation events (Mirzabekov, Trends in Biotechnology (1994) 12.27–32). Many of these hybidisation events are detected using fluorescent labels attached to nucleotides, the labels being detected using a sensitive fluorescent detector, e.g. a charge-coupled detector (CCD). The major disadvantages of these methods are that it is not possible to sequence long stretches of DNA, and that repeat sequences can lead to ambiguity in the results. These problems are recognised in Automation Technologies for Genome Characterisation, Wiley-Interscience (1997), ed T J Beugelsdijk, Chapter 10 205–225.

In addition, the use of high-density arrays in a multi-step analysis procedure can lead to problems with phasing. Phasing problems result from a loss in the synchronisation of a reaction step occurring on different molecules of the array. If some of the arrayed molecules fail to undergo a step in the procedure, subsequent results obtained for these molecules will no longer be in step with results obtained for the other arrayed molecules. The proportion of molecules out of phase will increase through successive steps and consequently the results detected will become ambiguous. This problem is recognized in the sequencing procedure described in U.S. Pat. No. 5,302,509.

An alternative sequencing approach is disclosed in EP-A-0381693, which comprises hybridising a fluorescently-labelled strand of DNA to a target DNA sample suspended in a flowing sample stream, and then using an exonuclease to cleave repeatedly the end base from the hybridised DNA. The cleaved bases are detected in sequential passage through a detector, allowing reconstruction of the base sequence of the DNA. Each of the different nucleotides has a distinct fluorescent label attached which is detected by laser-induced fluorescence. This is a complex method, primarily because it is difficult to ensure that every nucleotide of the DNA strand is labelled and that this has been achieved with high fidelity to the original sequence.

WO-A-96/27025 is a general disclosure of single molecule arrays. Although sequencing procedures are disclosed, there is little description of the applications to which the arrays can be applied. There is also only a general discussion on how to prepare the arrays.

SUMMARY OF THE INVENTION

According to the present invention, a device comprises a high density array of molecules capable of interrogation and immobilised on a solid generally planar source, wherein the array allows the molecules to be individually resolved by optical microscopy, and wherein each molecule is immobilised by covalent bonding to the surface, other than at that part of each molecule that can be interrogated.

According to a second aspect of the invention, a device comprises a high density array of relatively short molecules and relatively long polynucleotides immobilised on the surface of a solid support, wherein the polynucleotides are at a density that permits individual resolution of those parts that extend beyond the relatively short molecules. In this aspect, the shorter molecules can prevent non-specific binding of reagents to the solid support, and therefore reduce background interference.

According to a third aspect of the invention, a device comprises an array of polynucleotide molecules immobilised on a solid surface, wherein each molecule comprises a polynucleotide duplex linked via a covalent bond to form a hairpin loop structure, one end of which comprises a target polynucleotide, and the array has a surface density which allows the target polynucleotides to be individually resolved. In this aspect, the hairpin structures act to tether the target to a primer polynucleotide. This prevents loss of the primer-target during the washing steps of a sequencing procedure. The hairpins may therefore improve the efficiency of the sequencing procedures.

The arrays of the present invention comprise what are effectively single molecules. This has many important benefits for the study of the molecules and their interaction with other biological molecules. In particular, fluorescence events occuring on each molecule can be detected using an optical microscope linked to a sensitive detector, resulting in a distinct signal for each molecule.

When used in a multi-step analysis of a population of single molecules, the phasing problems that are encountered using high density (multi-molecule) arrays of the prior art, can be reduced or removed. Therefore, the arrays also permit a massively parallel approach to monitoring fluorescent or other events on the molecules. Such massively parallel data acquisition makes the arrays extremely useful in a wide range of analysis procedures which involve the screening/characterising of heterogeneous mixtures of molecules. The arrays can be used to characterise a particular synthetic chemical or biological moiety, for example in screening for particular molecules produced in combinatorial synthesis reactions.

The arrays of the present invention are particularly suitable for use with polynucleotides as the molecular species. The preparation of the arrays requires only small amounts of polynucleotide sample and other reagents, and can be carried out by simple means. Polynucleotide arrays according to the invention permit massively parallel sequencing chemistries to be performed. For example, the arrays permit simultaneous chemical reactions on and analysis of many individual polynucleotide molecules. The arrays are therefore very suitable for determining polynucleotide sequences.

An array of the invention may also be used to generate a spatial addressable array of single polynucleotide molecules. This is the simple consequence of sequencing the array. Particular advantages of such a spatially addressable array include the following.

1) Polynucleotide molecules on the array may act as identifier tags and may only need to be 10–20 bases long, and the efficiency required in the sequencing steps may only need to be better than 50%, as there will be no phasing problems.

2) The arrays may be reusable for screening once created and sequenced. All possible sequences can be produced in a very simple way, e.g. compared to a high density multi-molecule DNA chip made using photolithography.

DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic representation of apparatus that may be used to image arrays of the present invention;

FIG. **2** illustrates the immobilisation of a polynucleotide to a solid surface via a microsphere;

FIG. **3** shows a fluorescence time profile from a single fluorophore-labelled oligonucleotide, with excitation at 514 nm and detection at 600 nm;

FIG. **4** shows fluorescently labelled single molecule DNA covalently attached to a solid surface, and

FIG. **5** shows images of surface bound oligonucleotides hybridised with the complementary sequence.

DESCRIPTION OF THE INVENTION

According to the present invention, the single molecules immobilised onto the surface of a solid support should be capable of being resolved by optical means. This means that within the resolvable area of the particular imaging device used, there must be one or more distinct images each representing one molecule. Typically, the molecules of the array are resolved using a single molecule fluorescence microscope equipped with a sensitive detector, e.g. a charge-coupled detector (CCD). Each molecule of the array may be analysed simultaneously or, by scanning the array, a fist sequential analysis can be performed.

The molecules of the array are typically DNA, RNA or nucleic acid mimics, e.g. DNA or 2'-O-Meth-RNA. However, any other biomolecules, including peptides, polypeptides and other organic molecules, may be used. The molecules are formed on the array to allow interaction with other "cognate" molecules. It is therefore important to immobilise the molecules so that the portion of the molecule not physically attached to solid support is capable of being interrupted by a cognate. In some applications all the molecules in the single array will be the same, and may be used to interrogate molecules that are largely distinct. In other applications, the molecules on the array may all, or substantially all, be different, e.g. less than 50%, preferably less than 30% of the molecules will be the same.

The term "single molecule" is used herein to distinguish from high density multi-molecule arrays in the prior art, which may comprise distinct clusters of many molecules of the same type.

The term "individually resolved" is used herein to indicate that, when visualised, it is possible to distinguish one molecule on the array from its neighbouring molecules. Visualisation may be effected by the use of reporter labels, e.g. fluorophores, the signal of which is individually resolved.

The term "cognate molecule" is used herein to refer to any molecule capable of interacting, or interrogating, the arrayed molecule. The cognate may be a molecule that binds specifically to the arrayed molecule, for example a complementary polynucleotide, in a hybridisation reaction.

The term "interrogate" is used herein to refer to any interaction of the arrayed molecule with any other molecule. The interaction may be covalent or non-covalent.

The terms "arrayed polynucleotides" and "polynucleotide arrays" are used herein to define a plurality of single molecules that are characterised by comprising a polynucleotide. The term is intended to include the attachment of other molecules to a solid surface, the molecules having a polynucleotide attached that can be further interrogated. For example, the arrays may comprise protein molecules immobilised on a solid surface, the protein molecules being conjugated or otherwise bound to a short polynucleotide molecule that may be interrogated, to address the array.

The density of the arrays is not critical. However, the present invention can make use of a high density of single molecules, and these are preferable. For example, arrays with a density of 10^6–10^9 molecules per cm^2 may be used. Preferably, the density is at least $10^7/cm^2$ and typically up to $10^8/cm^2$. These high density arrays are in contrast to other arrays which may be described in the art as "high density"

but which are not necessarily as high and/or which do not allow single molecule resolution.

Using the methods and apparatus of the present invention, it may be possible to image at least 10^7 or 10^8 molecules simultaneously. Fast sequential imaging may be achieved using a scanning apparatus; shifting and transfer between images may allow higher numbers of molecules to be imaged.

The extent of separation between the individual molecules on the array will be determined, in part, by the particular technique used to resolve the individual molecule. Apparatus used to image molecular arrays are known to those skilled in the art. For example, a confocal scanning microscope may be used to scan the surface of the array with a laser to image directly a fluorophore incorporated on the individual molecule by fluorescence. This may be achieved using the apparatus illustrated in FIG. **1**, FIG. **1** shows a detector **1** at bandpass filter **2**, a pinhole **3**, a mirror **4** a laser beams **5** a dichroic mirror **6**, an objective **7**, a glass coverslip **8** and a sample **9** under study. Alternatively, a sensitive 2-D detector, such as a charge-coupled detector, can be used to provide a 2-D image representing the individual molecules on the array.

Resolving single molecules on the array with a 2-D detector can be done it at 100× magnification, adjacent molecules are separated by a distance of approximately at least 250 nm, preferably at least 300 nm and more preferably at least 350 nm. It will be appreciated that these distances are dependent on magnification, and flat other values can be determined accordingly, by one of ordinary skill in the art.

Other techniques such as scanning near-field optical microscopy (SNOM) are available which are capable of greater optical resolution, thereby permitting more dense arrays to be used. For example, using SNOM, adjacent molecules may be separated by a distance of less than 100 nm, e.g. 10 nm. For a description of scanning near-field optical microscopy, see Moyer et al, Laser Focus World (1993) 29(10).

An additional technique that may be used is surface-specific total internal reflection fluorescence microscopy (TIRFM); see, for example, Vale et al., Nature, (1996) 380. 451–453). Using this technique, it is possible to achieve wide-field imaging (up to 100 μm×100 μm) with single molecule sensitivity. This may allow arrays of greater than 10^7 resolvable molecules per cm^2 to be used.

Additionally, the techniques of scanning tunnelling microscopy (Binnig et al, Helvetica Physica Acta (1982) 55:726–735) and atomic force microscopy (Hansma, Ann. Rev Biophys Biomol Struct (1994) 23 115–139) are suitable for imaging the arrays of the present invention. Other devices which do not rely on microscopy may also be used, provided that they are capable of imaging within discrete areas on a solid support.

Single molecules may be arrayed by immobilisation to the surface of a solid support. This may be carried out by any known technique, provided that suitable conditions are used to ensure adequate separation of the molecules. Generally the array is produced by dispensing small volumes of a sample containing a mixture of molecules onto a suitably prepared solid surface, or by applying a dilute solution to the solid surface to generate a random array. In this manner, a mixture of different molecules may be arrayed by simple means. The formation of the single molecule array then permits interrogation of each arrayed molecule to be carried out.

Suitable solid supports are available commercially, and will be apparent to the skilled person. The supports may be manufactured from materials such as glass, ceramics, silica and silicon. The supports usually comprise a flat (planar) surface, or at least an array in which the molecules to be interrogated are in the same plane. Any suitable size may be used. For example, the supports might be of the order of 1–10 cm in each direction.

It is important to prepare the solid support under conditions which minimise or avoid the presence of contaminants. The solid support must be cleaned thoroughly, preferably with a suitable detergent, e.g. Decon-90, to remove dust and other contaminants.

Immobilisation may be by specific covalent or non-covalent interactions. Covalent attachment is preferred. If the molecule is a polynucleotide, immobilisation will preferably be at either the 5' or 3' position so that the polynucleotide is attached to the solid support at one end only. However, the polynucleotide may be attached to the solid support at any position along its length, the attachment acting to tether the polynucleotide to the solid support. The immobilised polynucleotide is then able to undergo interactions with other molecules or cognates at positions distant from the solid support. Typically the interaction will be such that it is possible to remove any molecules bound to the solid support through non-specific interactions e.g. by washing. Immobilisation in this manner results in well separated single molecules. The advantage of this is that it prevents interaction between neighbouring molecules on the array, which may hinder interrogation of the array.

In one embodiment of the invention, the surface of a solid support is first coated with streptavidin or avidin, and then a dilute solution of a biotinylated molecule is added at discrete sites on the surface using, for example, a nanoliter dispenser to deliver one molecule on average to each site.

In a preferred embodiment of the invention, The solid surface is coated with an epoxide and the molecules are coupled via an amine linkage. It is also preferable to avoid or reduce salt present in the solution containing the molecule to be arrayed. Reducing the salt concentration minimises the possibility of the molecules aggregating in the solution, which may affect the positioning on the array.

If the molecule is a polynucleotide, then immobilisation may be via hybridisation to a complementary nucleic acid molecule previously attached to a solid support. For example, the surface of a solid support may be first coated with a primer polynucleotide at discrete sites on the surface. Single-stranded polynucleotides are then brought into contact with the arrayed primers under hybridising conditions and allowed to "self-sort" onto the array. In this way, the arrays may be used to separate the desired polynucleotides from a heterogeneous sample of polynucleotides.

Alternatively, the arrayed primers may be composed of double-stranded polynucleotides with a single-stranded overhang ("sticky-ends"). Hybridisation with target polynucleotides is then allowed to occur and a DNA ligase used to covalently link the target DNA to the primer. The second DNA strand can then be removed under melting conditions to leave an arrayed polynucleotide.

In an embodiment of the invention, the target molecules are immobilised onto non-fluorescent streptavidin or avidin-functionalised polystyrene latex microspheres, as shown in FIG. **2**. FIG. **2** shows a microsphere **11**, a streptavidin molecule **12**, a biotin molecule **13** and a fluorescently labelled polynucleotide **14**. The microspheres are immobilised in turn onto a solid support to fix the target sample for microscope analysis. Alternative microspheres suitable for use in the present invention are well known in the art.

In one aspect of the present invention, the devices comprise arrayed polynucleotides, each polynucleotide comprising a hairpin loop structure, one end of which comprises a target polynucleotide, the other end comprising a relatively short polynucleotide capable of acting as a primer in the polymerase reaction. This ensures that the primer is able to perform its priming function during a polymerase-based sequencing procedure, and is not removed during any washing step in the procedure. The target polynucleotide is capable of being interrogated.

The term "hairpin loop structure" refers to a molecular stem and loop structure formed from the hybridisation of complementary polynucleotides that are covalently linked. The stem comprises the hybridised polynucleotides and the loop is the region that covalently links the two complementary polynucleotides. Anything from a 10 to 20 (or more) base pair double-stranded (duplex) region may be used to form the stem. In one embodiment, the structure may be formed from a single-stranded polynucleotide having complementary regions. The loop in this embodiment may be anything from 2 or more non-hybridised nucleotides. In a second embodiment, the structure is formed from two separate polynucleotides with complementary regions, the two polynucleotides being linked (and the loop being at least partially formed) by a linker moiety. The linker moiety forms a covalent attachment between the ends of the two polynucleotides. Linker moieties suitable for use in this embodiment will be apparent to the skilled person. For example, the linker moiety may be polyethylene glycol (PEG).

There are many different ways of forming the hairpin structure to incorporate the target polynucleotide. However, a preferred method is to form a first molecule capable of forming a hairpin structure, and ligate the target polynucleotide to this. Ligation may be carried out either prior to or after immobilisation to the solid support. The resulting structure comprises the single-stranded target polynucleotide at one end of the hairpin and a primer polynucleotide at the other end.

In one embodiment, the target polynucleotide is genomic DNA purified using conventional methods. The genomic DNA may be PCR-amplified or used directly to generate fragments of DNA using either restriction endonucleases, other suitable enzymes, a mechanical form of fragmentation or a non-enzymatic chemical fragmentation method. In the case of fragments generated by restriction endonucleases, hairpin structures bearing a complementary restriction site at the end of the first hairpin may be used, and selective ligation of one strand of the DNA sample fragments may be achieved by one of two methods.

Method 1 uses a first hairpin whose restriction site contains a phosphorylated 5' end. Using this method, it may be necessary to first de-phosphorylate the restriction-cleaved genomic or other DNA fragments prior to ligation such that only one sample strand is covalently ligated to the hairpin.

Method 2, in the design of the hairpin, a single (or more) base gap can be incorporated at the 3' end (the receded strand) such that upon ligation of the DNA fragments only one strand is covalently joined to the hairpin. The base gap can be formed by hybridising a further separate polynucleotide to the 5'-end of the first hairpin structure. On ligation, the DNA fragment has one strand joined to the 5'-end of the first hairpin, and the other strand joined to the 3'-end of the further polynucleotide. The further polynucleotide (and the other strand of the DNA fragment) may then be removed by disrupting hybridization.

In either case, the net result should be covalent ligation of only one strand of a DNA fragment of genomic or other DNA, to the hairpin. Such ligation reactions may be carried out in solution at optimised concentrations based an conventional ligation chemistry, for example, carried out by DNA ligases or non-enzymatic chemical ligation. Should the fragmented DNA be generated by random shearing of genomic DNA or polymerase, then the ends can be filled in with Klenow fragment to generate blunt-ended fragments which may be blunt-end-ligated onto blunt-ended hairpins. Alternatively, the blunt-ended DNA fragments may be ligated to oligonucleotide adapters which are designed to allow compatible ligation with the sticky-end hairpins, in the manner described previously.

The hairpin-ligated DNA constructs may then be covalently attached to the surface of a solid support to generate a single molecule array (SMA), or ligation may follow attachment to form the array.

The arrays may then be used in procedures to determine the sequence of the target polynucleotide. If the target fragments are generated via restriction digest of genomic DNA, the recognition sequence of the restriction or other nuclease enzyme will provide 4, 6, 8 bases or more of known sequence (dependent on the enzyme). Further sequencing of between 10 and 20 bases on the SMA should provide sufficient overall sequence information to place that stretch of DNA into unique context with a total human genome sequence, thus enabling the sequence information to be used for genotyping and more specifically single nucleotide polymorphism (SNP) scoring.

Simple calculations have suggested the following based on sequencing a 10^7 molecule SMA prepared from hairpin ligation. For a 6 base pair recognition sequence, a single restriction enzyme will generate approximately 10^4 ends of DNA. If a stretch of 13 bases is sequenced on the SMA (i.e 13×10^6 bases), approximately 13,000 SNPs will be detected. One application of such a sample preparation and sequencing format would in general be for SNP discovery in pharmaco-genetic analysis. The approach is therefore suitable for forensic analysis or any other system which requires umambiguous identification of individuals to a level as low 10^3 SNPs.

It is of course possible to sequence the complete target polynucleotide, if required.

In a separate aspect of the invention, the devices may comprise immobilised polynucleotides and other immobilised molecules. The other molecules are relatively short compared to the polynucleotides and are intended to prevent non-specific attachment of reagents, e.g. fluorophores, with the solid support, thereby reducing background interference. In one embodiment, the other molecules are relatively short polynucleotides. However, many different molecules may be used, e.g. peptides, proteins, polymers and synthetic chemicals, as will be apparent to the skilled person. Preparation of the devices may be carried out by first preparing a mixture of the relatively long polynucleotides and of the relatively short molecules. Usually, the concentration of the latter will be in excess of that of the long polynucleotides. The mixture is then placed in contact with a suitably prepared solid support, to allow immobilisation to occur.

The single molecule arrays have many applications in methods which rely on the detection of biological or chemical interactions with arrayed molecules. For example, the arrays may be used to determine the properties or identities of cognate molecules. Typically, interaction of biological or chemical molecules with the arrays are carried out in solution.

In particular, the arrays may be used in conventional assays which rely on the detection of fluorescent labels to obtain information on the arrayed molecules. The arrays are particularly suitable for use in multi-step assays where the loss of synchronisation in the steps was previously regarded as a limitation to the use of arrays. When the arrays are composed of polynucleotides they may be used in conventional techniques for obtaining genetic sequence information. Many of these techniques rely on the stepwise identification of suitably labelled nucleotides, referred to in U.S. Pat. No. 5,634,413 as "single base" sequencing methods.

In an embodiment of the invention, the sequence of a target polynucleotide is determined in a similar manner to that described in U.S. Pat. No. 5,634,413, by detecting the incorporation of nucleotides into the nascent strand through the detection of a fluorescent label attached to the incorporated nucleotide. The target polynucleotide is primed with a suitable primer (or prepared as a hairpin construct which will contain the primer as part of the hairpin), and the nascent chain is extended in a stepwise manner by the polymerase reaction. Each of the different nucleotides (A, T, G and C) incorporates a unique fluorophore at the 3' position which acts as a blocking group to prevent uncontrolled polymerization. The polymerase enzyme incorporates a nucleotide into the nascent chain complementary to the target, and the blocking group prevents further incorporation of nucleotides. The array surface is then cleared of unincorporated nucleotides and each incorporated nucleotide is "read" optically by a charge-coupled detector using laser excitation and filters. The 3'-blocking group is then removed (deprotected), to expose the nascent chain for further nucleotide incorporation.

Because the array consists of distinct optically resolvable polynucleotides, each target polynucleotide will generate a series of distinct signals as the fluorescent events are detected. Details of the full sequence are then determined.

The number of cycles that can be achieved is governed principally by the yield of the deprotection cycle. If deprotection fails in one cycle, it is possible that later deprotection and continued incorporation of nucleotides can be detected during the next cycle. Because the sequencing is performed at the single molecule level, the sequencing can be carried out on different polynucleotide sequences at one time without the necessity for separation of the different sample fragments prior to sequencing. This sequencing also avoids the phasing problems associated with prior art methods.

Deprotection may be carried out by chemical, photochemical or enzymatic reactions.

A similar, and equally applicable, sequencing method is disclosed in EP-A-0640146.

Other suitable sequencing procedures will be apparent to the skilled person. In particular, the sequencing method may rely on the degradation of the arrayed polynucleotides, the degradation products being characterised to determine the sequence.

An example of a suitable degradation technique is disclosed in WO-A-95/20053, whereby bases on a polynucleotide are removed sequentially, a predetermined number at a time though the use of labeled adaptors specific for the bases, and a defined exonuclease cleavage.

A consequence of sequencing using non-destructive methods is that it is possible to form a spatially addressable away for further characterisation studies, and therefore non-destructive sequencing may be preferred. In this context, term "spatially addressable" is used herein to describe how different molecules may be identified on the basis of their position on an array.

Once sequenced, the spatially addressed arrays may be used in a variety of procedures which require the characterisation of individual molecules from heterogeneous populations.

One application is to use the arrays to characterise products synthesised in combinatorial chemistry reactions. During combinatorial synthesis reactions, it is usual for a tag or label to be incorporated onto a beaded support or reaction product for the subsequent characterisation of the product. This is adapted in the present invention by using polynucleotide molecules as the tags, each polynucleotide being specific for a particular product and using the tags to hybridise onto a spatially addressed array. Because the sequence of each arrayed polynucleotide has been determined previously, the detection of a hybridisation event on the array reveals the sequence of the complementary tag on the product. Having identified the tag, it is then possible to confirm which product this relates to. The complete process is therefore quick and simple, and the arrays may be reused for high through-put screening. Detection may be carried out by attaching a suitable label to the product, e.g. a fluorophore.

Combinatorial chemistry reactions may be used to synthesise a diverse range of different molecules, each of which may be identified using the addressed arrays of the present invention. For example, combinatorial chemistry may be used to produce therapeutic proteins or peptides that can be bound to the arrays to produce an addressed array of target proteins. The targets may then be screened for activity, and those proteins exhibiting activity may be identified by their position on the array as outlined above.

Similar principles apply to other products of combinatorial chemistry, for example the synthesis of non-polymeric molecules of m.wt<1000. Methods for generating peptides/proteins by combinatorial methods are disclosed in U.S. Pat. No. 5,643,768 and U.S. Pat. No. 5,658,754. Split-and-mix approaches may also be used, as described in Nielsen et al. J Am Chem Soc (1993) 115.9812–9813.

In an alternative approach, the products of the combinatorial chemistry reactions may comprise a second polynucleotide tag not involved in the hybridisation to the array. After formation by hybridisation, the array may be subjected to repeated polynucleotide sequencing to identify the second tag which remains free. The sequencing may be carried out as described previously.

Therefore, in this application, it is the tag that provides the spatial address on the array. The tag may then be removed from the product by, for example, a cleavable linker, to leave an untagged spatially addressed array.

A further application is to display proteins via an immobilised polysome containing trapped polynucleotides and protein in a complex, as described in U.S. Pat. Nos. 5,643,768 and 5,658,754.

In a separate embodiment of the invention, the arrays maybe used to characterise an organism. For example, an organism's genomic DNA may be screened using the arrays, to reveal discrete hybidisation patterns that are unique to an individual. This embodiment may therefore be likened to a "bar code" for each organism. The organism's genomic DNA may be first fragmented and detectably-labelled, for example with a fluorophore. The fragmented DNA is then applied to the array under hybridising conditions and any hybridisation events monitored.

Alternatively, hybridisation may be detected using an in-built fluorescence based detection system in the arrayed molecule, for example using the "molecular beacons" described in Nature Biotechnology (1996) 14.303–308.

It is possible to design the arrays so that the hybridisation pattern generated is unique to the organism and so could be used to provide valuable information on the genetic character of an individual. This may have many useful applications in forensic science. Alternatively, the methods may be carried out for the detection of mutations or allelic variants within the genomic DNA of an organism.

For genotyping, it is desirable to identify if a particular sequence is present in the genome. The smallest possible unique oligomer is a 16-mer (assuming randomness of the genome sequence), i.e. statistically there is a probability of any given 16-base sequence occurring only once in the human genome (which has 3×10^9 bases). There are c.4×10^9 possible 16-mers which would fit within a region of 2 cm×2 cm (assuming a single copy at a density of 1 molecule per 250 nm×250 nm square). It is therefore necessary to determine only if a particular 16-mer is present or not, and so quantitative measurements are unnecessary. Identifying a mutation in a particular region and what the mutation is can be carried out using the 16-mer library. Mapping back onto the human genome would be possible using published data and would not be a problem once the entire genome has been determined. There is built-in self-check, by looking at the hybridisation to particular 16-mers so that if there is a single point mutation, this will show up in 16 different 16-mers, identifying a region of 32 bases in the genome (the mutation would occur at the top of one 16-mer and then at the second base in a related 16-mer etc). Thus, a single point mutation would result in 16 of the 16-mers not showing hybridisation and a new set of 16 showing hybridisation plus the same thing for the complementary strand. In summary, considering both strands of DNA, a single point mutation would result in 32 of the 16-mers not showing hybridisation and 32 new 16-mers showing hybridisation, i e quite large changes on the hybridisation pattern to the array.

By way of example, a sample of human genomic DNA may be restriction-digested to generate short fragments, then labelled using a fluorescently-labelled monomer and a DNA polymerase or a terminal transferase enzyme. This produces short lengths of sample DNA with a fluorophore at one end. The melted fragments may then be exposed to the array and the pixels where hybridisation occurs or not would be identified. This produces a genetic bar code for the individual with (if oligonucleotides of length 16 were used) c 4×10^9 binary coding elements. This would uniquely define a person's genotype for pharmagenomic applications. Since the arrays should be reusable, the same process could be repeated on a different individual.

In one embodiment of the invention, a method for determining a single nucleotide polymorphism (SNP) present in a genome comprises immobilising fragments of the genome onto the surface of a solid support to form an army as defined above, identifying nucleotides at selected positions in the genome, and comparing the results with a known consensus sequence to identify any differences between the consensus sequence and the genome. Identifying the nucleotides at selected positions in the genome may be carried out by contacting the array sequentially with each of the bases A, T, G and C, under conditions that permit the polymerase reaction to proceed, and monitoring the incorporation of a base at selected positions in the complementary sequence.

The fragments of the genome may be unamplified DNA obtained from several cells from an individual, which is treated with a restriction enzyme. As indicated above, it is not necessary to determine the sequence of the full fragment. For example, it may be preferable to determine the sequence of 16–30 specific bases, which is sufficient to identify the DNA fragment by comparison to a consensus sequence, e.g. to that known from the Human Genome Project. Any SNP occurring within the sequenced region can then be identified. The specific bases do not have to be contiguous. For example, the procedure may be carried out by the incorporation of non-labelled bases followed, at pre-determined positions, by the incorporation of a labeled base. Provided that the sequence of sufficient bases is determined, it should be possible to identify the fragment. Again, any SNPs occurring at the determined base positions, can be identified. For example, the method may be used to identify SNPs that occur after cytosine. Template DNA (genomic fragments) can be contacted with each of the bases A, T and G, added sequentially or together, so that the complementary strand is extended up to a position that requires C. Non-incorporated bases can then be removed from the array, followed by the addition of C. The addition of C is followed by monitoring the next base incorporation (using a labelled base). By repeating this process a sufficient number of times, a partial sequence is generated where each base immediately following a C is known. It will then be possible to identify the full sequence by comparison of the partial sequence to a reference sequence. It will then also be possible to determine whether there are any SNPs occurring after any C.

To further illustrate this, a device may comprise 10^7 restriction fragments per cm^2. If 30 bases are determined for each fragment, this means 3×10^8 bases are identified. Statistically, this should determine 3×10^5 SNPs for the experiment. If the fragments each comprise 1000 nucleotides, it is possible to have 10^{10} nucleotides per cm^2, or three copies of the human genome. The approach therefore permits large sequence or SNP analysis to be performed.

Viral and bacterial organisms may also be studied, and screening nucleic acid samples may reveal pathogens present in a disease, or identify microorganisms in analytical techniques. For example, pathogenic or other bacteria may be identified using a series of single molecule DNA chips produced from different strains of bacteria. Again, these chips are simple to make and reusable.

In a further example, double-stranded arrays may be used to screen protein libraries for binding, using fluorescently labelled proteins. This may determine proteins that bind to a particular DNA sequence, i e proteins that control transcription. Once the short sequence that the protein binds to has been determined, it may be made and affinity purification used to isolate and identify the protein. Such a method could find all the transcription-controlling proteins. One such method is disclosed in Nature Biotechnology (1999) 17.573–577.

Another use is in expression monitoring. For this, a label is required for each gene. There are approximately 100,000 genes in the human genome. There are 262,144 possible 9-mers, so this is the minimum length of oligomer needed to have a unique tag for each gene. This 9-mer label needs to be at a specific point in the DNA and the best point is probably immediately after the poly-A tail in the mRNA (i e a 9-mer linked to a poly-T guide sequence). Multiple copies of these 9-mers should be present, to permit quantitation of gene expression. 100 copies would allow determination of relative expression from 1–100%. 10,000 copies would allow determination of relative gene expression from 0.01–100%. 10,000 copies of 262,144 9-mers would fit inside 1 cm×1 cm at close to maximum density.

The use of nanovials in conjunction with any of the above methods may allow a molecule to be cleaved from the surface, yet retain its spatial integrity. This permits the generation of spatially addressable arrays of single mol-

ecules in free solution, which may have advantages where the surface attachment impedes the analysis (e.g drug screening). A nanovial is a small cavity in a flat glass surface, e.g. approx 20 μm in diameter and 10 μm deep. They can be placed every 50 μm, and so the array would be less dense than a surface-attached array; however, this could be compensated for by appropriate adjustment in the imaging optics.

The following Examples illustrate the invention, with reference to the accompanying drawings.

EXAMPLE 1

The microscope set-up used in the following Example was based on a modified confocal fluorescence system using a photon detector as shown in FIG. **1**. Briefly, a narrow, spatially filtered laser beam (CW Argon ton Laser Technology RPC50) was passed through an acousto-optic modulator (AOM) (A A Opto-Electronic) which acts as a fast optical switch. The acousto-optic modulator was switched on and, the laser beam was directed through an oil emersion objective (100×NA=1 3) of an inverted optical microscope (Nikon Diaphor 200) by a dichroic beam splitter (540DRLP02 or 505DRLP02, Omega Optics Inc). The objective focuses the light to a diffraction-limited spot on the target sample immobilised on a thin glass coverslip. Fluorescence from the sample was collected by the same objective, passed through the dichroic beam splitter and directed though a 50 μm pinhole (Newport Corp.) placed in the image plane of the microscope observation port. The pinhole rejects light emerging from the sample which is out of the plane of the laser scatter. The remaining fluorescence was separated spectrally by a dichroic beam splitter into red and green components which was filtered to remove residual laser scatter. The remaining fluorescence components were then focused onto separate single photon avalanche diode detectors and the signals recorded onto a multichannel scalar (MCS) (MCS-Plus, E G & G Ortec) with time resolutions in the 1 to 10 ms range.

The target sample was a 5'-biotin-modified 13-mer primer oligonucleotide prepared using conventional phosphoramidite chemistry, and having SEQ ID No 1 (see listing, below). The oligonucleotide was post-synthetically modified by reaction of the uridine base with the succinimdyl ester of tetramethylrhodamine (TMR).

Glass coverslips were prepared by cleaning with acetone and drying under nitrogen. A 50 μl aliquot of biotin-BSA (Sigma) redissolved in PBS buffer (0.01 M, pH 7.4) at 1 mg/ml concentration was deposited on the clean coverslip and incubated for 8 hours at 30° C. Excess biotin-BSA was removed by washing 5 times with MilliQ water and drying under nitrogen. Non-fluorescent streptavidin functionalised polystyrene latex microspheres of diameter 500 nm (Polysciences Inc) were diluted in 100 mM NaCl to 0 1 solids and deposited as a 1 μl drop on the biotinylated coverslip surface. The spheres were allowed to dry for one hour and unbound beads removed by washing 5 times with MilliQ water. This procedure resulted in a surface coverage of approximately 1 sphere/100 μm×100 μm.

The non-fluorescent microspheres were found to have a broad residual fluorescence at excitation wavelength 514 nm, probably arising from small quantities of photoactive constituents used in the colloidal preparation of the microspheres. The microspheres were therefore photobleached by treating the prepared coverslip in a laser beam of a frequency doubled (532 nm) Nd.YAG pulsed dye laser, for 1 hour.

The biotinylated I3-TMR ssDNA was coupled to the streptavidin functionalised microspheres by incubating a 50 μl sample of 0.1 pM DNA (diluted in 100 mM NaCl, 100 mM Tris) deposited over the microspheres. Unbound PNA was removed by washing the coverslip surface 5 times with MilliQ water.

Low light level illumination from the microscope condenser was used to position visually a microsphere at 10× magnification so that when the laser was switched on the sphere was located in the centre of the diffraction limited focus. The condenser was then turned off and the light path switched to the fluorescence detection port. The MCS was initiated and the fluorescence omitted from the latex sphere recorded on one or both channels. The sample was excited at 514 nm and detection was made on the 600 nm channel.

FIG. **3** shows clearly that the fluorescence is switched on as the laser is deflected into the microscope by the AOM, 0 5 seconds after the start of a scan. The intensity of the fluorescence remains relatively constant for a short period of time (100 ms–3s) and disappears in a single step process. The results show that single molecule detection is occurring. This single step photobleaching is unambiguous evidence that the fluorescence is from a single molecule.

EXAMPLE 2

This Example illustrates the preparation of single molecule arrays by direct covalent attachment to glass followed by a demonstration of hybridisation to the array.

Covalently modified slides were prepared as follows. Spectrosil-2000 slides (TSL, UK) were rinsed in milli-Q to remove any dust and placed wet in a bottle containing near Decon-90 and left for 12 h at room temperature. The slides were rinsed with milli-Q and placed in a bottle containing a solution of 1.5% glycidoxypropyltrimethoxy-silane in milli-Q and magnetically stirred for 4 h at room temperature rinsed with milli-Q and dried under N_2 to liberate an epoxide coated surface.

The DNA used was that shown in SEQ ID No. 2 (see sequence listing below), where n represents a 5-methyl cytosine (Cy5) with a TMR group coupled via a link to the n4 position.

A sample of this (5 μl, 450 pM) was applied as a solution in neat milli-Q.

The DNA reaction was left for 12 h at room temperature in a humid atmosphere to couple to the epoxide surface. The slide was then rinsed with milli-Q and dried under N_2.

The prepared slides can be stored wrapped in foil in a desiccator for at least a week without any noticeable contamination or loss of bound material. Control DNA of the same sequences and fluorophore but without the 5'-amino group shows little stable coverage when applied at the same concentration.

The TMR labelled slides were then treated with a solution of complementary DNA (SEQ D No. 3) (5 μM, 10 μl) in 100 nM PBS. The complementary DNA has the sequence shown in SEQ ID No. 3, where n represents a methylcytosine group.

After 1 hour at room temperature the slides were cooled to 4° C. and left for 24 hours. Finally, the slides were washed in PBS (100 mM, 1 mL) and dried under N_2.

A chamber was constructed on the slide by sealing a coverslip (No 0, 22×22 mm, Chance Propper Ltd, UK) over the sample area on two sides only with prehardened microscope mounting medium (Eukitt, O. Kindler GmbH & Co Freiburg, Germany) whilst maintaining a gap of less than 200 μm between slide and coverslip. The chamber was

flushed 3× with 100 μl PBS (100M NaCl) and allowed to stabilise for 5 minutes before analysing on a fluorescence microscope.

The slide was inverted so that the chamber coverslip contacted the objective lens of an inverted microscope (Nikon TE200) via an immersion oil interface. A 60° fused silica dispersion prism was optically coupled to the back of the slide through a thin film of glycerol. Laser light was directed at the prism such that at the glass/sample interface it subtends an angle of approximately 68° to the normal of the slide and subsequently undergoes total Internal Reflection (TIR). The critical angle for water interface is 66°.

Fluorescence from single molecules of DNA-TMR or DNA-Cy5 produced by excitation with the surface specific evanescent wave following TIR is collected by the objective lens of the microscope and imaged onto an intensified Charge Coupled Device (ICCD) camera (Pentamax, Princeton Instruments, N.J.). Two images were recorded using a combination of 1) 532 nm excitation (frequency doubled solid state Nd YAG, Antares, Coherent) with a 580 nm fluorescence (580DF30, Omega Optics, USA) filter for TMR and 2) 630 nm excitation (nd-YAG pumped dye laser, Coherent 700) with a 670 nm filter (670DF40, Omega Optics, USA) for Cy5. Images were recorded with an exposure time of 500 ms at the maximum gain of 10 on the ICCD. Laser powers incident at the prism were 50 mW and 40 mW at 532 nm and 630 nm respectively. A third image was taken with 532 nm excitation and detection at 670 nm to determine the level of cross talk from TMR on the Cy5 channel.

Single molecules were identified by single points of fluorescence with average intensities greater than 3× that of the background. Fluorescence from a single molecule is confined to a few pixels typically a 3×3 matrix at 100× magnification, and has a narrow Gaussian-like intensity profile. Single molecule fluorescence is also characterised by a one-step photobleaching process in the time course of the intensity and was used to distinguish single molecules from pixel regions containing two or more molecules, which exhibited multi-step processes. FIGS. **4***a* and **4***b* show 60 μm×60 μm fluorescence images from covalently modified slides with DNA-TMR starting concentrations of 45 pM and 450 pM. FIG. **4***c* shows a control slide which was treated as above but with DNA-TMR lacking the 5' amino modification.

To count molecules, a threshold for fluorescence intensities is first set to exclude background noise. For a control sample, the background is essentially the thermal noise of the ICCD measured to be 76 counts with a standard deviation of only 6 counts. A threshold is arbitrarily chosen as a linear combination of the background, the average counts over an image and the standard deviation over an image. In general, the latter two quantities provide a measure of the number of pixels and range of intensities above background. This method gives rise to threshold levels which are at least 12 standard deviations above the background with a probability of less than 1 in 144 pixels contributing from noise. By defining a single molecule fluorescent point as being at least a 2×2 matrix of pixels and no larger than a 7×7, the probability of a single background pixel contributing to the counting is eliminated and clusters are ignored.

In this manner, the surface density of single molecules of DNA-TMR is measured at 2.9×10^6 molecules/cm^2 (238 molecules in FIG. **4***a*) and 5 8×10^6 molecules/cm^2 (469 molecules in FIG. **4***b*) at 45 pM and 450 pM DNA-TMR coupling concentrations. The density is clearly not directly proportional to DNA concentration but will be some function of the concentration, the volume of sample applied, the area covered by the sample and the incubation time. The percentage of non-specifically bound DNA-TMR and impurities contribute of the order of 3–9% per image (8 non-specifically bound molecules in FIG. **4***c*). Analysis of the photobleaching profiles shows only 6% of fluorescence points contain more than 1 molecule.

Hybridisation was identified by the co-localisation of discreet points of fluorescence from single molecules of TMR and Cy-5 following the superposition of two images. FIGS. **5***a* and **5***b* show images of surface bound 20-mer labelled with TMR and the complementary 20-mer labelled with Cy-5 deposited from solution. FIG. **5***d* shows those fluorescent points that are co-localised on the two former images. The degree of hybridisation was estimated to be 7% of the surface-bound DNA (10 co-localised points in 141 points from FIGS. **5***d* and **5***a*, respectively). The percentage of hybridised DNA is estimated to be 37% of all surface-adsorbed DNA-Cy5 (10 co-localised points in 27 points from FIGS. **5***d* and **5***b*, respectively). Single molecules were counted by matching size and intensity of fluorescent points to threshold criteria which separate single molecules from background noise and cosmic rays. FIG. **5***d* shows the level of cross-talk from TMR on the Cy5 channel which is 2% as determined by counting only those fluorescent points which fall within the criteria for determining the TMR single molecule fluorescence (2 fluorescence points in 141 points from FIGS. **5***c* and **5***a*, respectively).

This Example demonstrates that single molecule arrays can be formed, and hybridisation events detected according to the invention. It is expected that the skilled person will realise that modifications may be made to improve the efficiency of the process. For example, improved washing steps, e g using a flow cell, would reduce background noise and permit more concentrated solutions to be used, and hybridisation protocols could be adapted by varying the parameters of temperature, buffer, time etc.

EXAMPLE 3

This experiment demonstrates the possibility of performing enzymatic incorporation on a single molecule array. In summary, primer DNA was attached to the surface of a solid support, and template DNA hybridised thereto. Two cycles of incorporation of fluorophore-labelled nucleotides was then completed. This was compared against a reference experiment where the immobilised DNA was pre-labelled with the same two fluorophores prior to attachment to the surface, and control experiments performed under adverse conditions for nucleotide incorporation.

The primer DNA sequence and the template DNA sequence used in this experiment are shown in SEQ ID NOS. 4 and 5, respectively.

The buffer used contained 4 mM $MgCl_2$, 2 mM DTT, 50 mM Tris HCl (pH 7 6) 10 mM NaCl and 1 mm K_2PO_3 (100 μl).

Preparation of Slides.

Silica slides were treated with decon for at least 24 hours and rinsed in water and EtOH directly before use. The dried slides were placed in a 50 ml solution of 2% glycidoxypropyltrimethoxysalane in EtOH/H_2SO_4 (2 drops/500 ml) at room temperature for 2 hours. The slides were then rinsed in EtOH from a spray bottle and dried under N_2. The DNA samples (SEQ to NO 4) were applied either as a 40–100 pM solution (5 μl) in 10 mM K_2PO_3 pH 7.6 (allowed to dry overnight), or at least 1 μM concentration over a sealed slide. The slides allowed to dry overnight were left over a layer of

water for 18 hours at room temperature and then rinsed with milli-q (approx. 30 ml from a spray bottle) and dried under N_2. The sealed slides were simply flushed with 50 ml buffer prior to use. Control slides with no coupled DNA were simply left under the buffer for identical time periods.

Enzyme Extensions on a Surface.

For the first incorporation cycle, samples were prepared with the buffer containing BSA (to 0.2 mg/ml), the triphosphate (Cy3dUTP; to 20 μM) and the polymerase enzyme (T4 exo-; to 500 nM). In certain experiments, the template DNA was also added at 2 μM. The mixture was flowed into cells which were incubated at 37° C. for 2 hours and flushed with 500 ml buffer. The second incorporation cycle with Cy5dCTP (20 μM), dATP (100 μm) and dGTP (100 μM) was performed in the same way. The cells were flushed with 50 ml buffer and left for 12 hours prior to imaging. Control reactions were performed as above with a) no DNA coupled prior to extension; b) DNA attached but no polymerase in the extension buffer, and c) DNA attached, but the polymerase denatured by boiling.

Reference Sample.

A reference sample, not immobilised to the surface, was prepared in the following way.

Buffer containing 1 μM of the sample DNA, BSA (0.2 mg/ml), TMR-labelled dUTP (20 μM) and the polymerase enzyme (T4 exo-, 500 nM; 100 μl) was prepared. The reaction was analysed and purified by reverse phase HPLC (5–30% acetonitrile in ammonium acetate over 30 min.) with UV and fluorescence detection in all cases, the labelled DNA was clearly separate from both the unlabelled DNA and the labelled dNTP's. The material was concentrated and dissolved in 10 mM K_2PO_3 for analysis by A260 and fluorescence. The material purified by HPLC was further extended with labelled dCTP (20 μM), dATP (100 μM) and dGTP (100 μM) and HPLC purified again. Surface coupling was then performed dry, at 100 μM concentrations.

Microscopic Analysis.

Following the single molecule DNA attachment procedure and extension reactions, the sample cells were analysed on a single molecule total internal reflection fluorescence microscope (TIRFM in the following manner. A 60° fused silica dispersion prism was coupled optically to the slide through an aperture in the cell via a thin film of glycerol. Laser light was directed at the prism such that at the glass/sample interface it subtends an angle of approximately 68° to the normal of the slide and subsequently undergoes total internal reflection. The critical angle for a glass/water interface is 66°. An evanescent field is generated at the interface which penetrates only ~150 nm into the aqueous phase. Fluorescence from single molecules excited within this evanescent field is collected by a 100× objective lens of an inverted microscope, filtered spectrally from the laser light and imaged onto an Intensified Charge Coupled Device (ICCD) camera.

Two 90 μm×90 μm images were recorded using a combination of: 1) 532 nm excitation (frequently doubled Nd.YAG) with a 580 nm interference filter for Cy5 detection, and 2) 630 nm excitation (Nd.YAG pumped DCM dye laser) with a 670 nm filter for Cy5 detection. Images were recorded with an exposure time of 500 ms at the maximum ICCD gain of 5.75 counts/photoelectron. Laser powers incident at the prism were 30 mW and 30 mW at 532 nm and 630 nm respectively. Two colour fluorophore labelled nucleotide incorporations are identified by the co-localisation of discrete points of fluorescence from single molecules of Cy3 and Cy5 following superimposing the two images. Molecules are considered co-localised when fluorescent points are within a pixel separation of each other. For a 90 μm×90 μm field, projected onto a CCD array of 512×512 pixels, the pixel size dimension is 0 176 μm.

Results. The results of the experiment are shown in Table 1. The values shown are an average of the number of molecules imaged (Cy3 and Cy5) over all frames (100 in each) compiled in each experiment and the number of those molecules which are co-localised.

The final column represents the number of co-localised molecules expected if the two fluorophores were randomly dispersed across the sample slide ($N \sim \pi\Delta\gamma$ where n is the surface density of molecules and $\Delta\gamma$=0 176 μm is the minimum measurable separation). The number in brackets indicates the magnitude by which the level of co-locations in each experiment is greater than random.

TABLE 1

System	Cy3	Cy5	Co-local	% of Total	Random
Reference	30	36	3	8	0.05 (×100)
Incorporation A	75	75	12	8	0.3 (×40)
Incorporation B	354	570	76	8	10 (×7.6)
No DNA	100	280	9	2	2 (×3.5)
No Enzyme	26	332	3	1	1/5 (×2)
Denatured T4	89	624	18	2.5	6 (×3)

The percentage of co-localisation observed on this sample represents the maximum measurable for a dual labelled system, i.e. there is a detection ceiling due to photophysical effects which means the level is not 100%. These effects may arise from interactions of the fluorophores with the DNA or the surface or both.

There is a statistically higher level of co-localisation in the incorporation experiments compared to the controls (8% versus 2% respectively). This shows that it is possible to perform enzymatic incorporation on the SMA and the level of incorporation is close to that of the reference sequence. Improvements in the surface attachment and the nature of the surface are required to increase the level of co-localisation in the reference and to increase the detection efficiency of the enzyme incorporation.

EXAMPLE 4

This Example illustrates the preparation of single molecule arrays by direct covalent attachment of hairpin loop structures to glass.

A solution of 1% glycidoxypropyltrimethoxy-silane in 95% ethanol/5% water with 2 drops H_2SO_4 per 500 ml was stirred for 5 minutes at room temperature. Clean, dry Spectrosil-2000 slides (TSL, UK) were placed in the solution and the stirring stopped. After 1 hour the slides were removed, rinsed with ethanol, dried under N_2 and oven-cured for 30 min at 100° C. These 'epoxide' modified slides were then treated with 1 μM of labelled DNA (5'-Cy3-CTGCT-GAAGCGTCGGCAGGT-heg-ami-nodT-heg-ACCTGC-CGACGCT-3') (SEQ ID NOS. 6 and 7) in 50 mM potassium phosphate buffer, pH 7.4 for 18 hours at room temperature and, prior to analysis, flushed with 50 mM potassium phosphate, 1 mM EDTA, pH 7.4. The coupling reactions were performed in sealed teflon blocks under a pre-mounted coverslip to prevent evaporation of the sample and allow direct imaging.

The DNA structure was designed as a self-priming template system with an internal amino group attached as an amino deoxy-thymidine held by two **18** atom hexaethylene

glycol (heg) spacers, and was synthesised by conventional DNA synthesis techniques using phosphoramidite monomers.

For imaging, one slide was inverted so that the chamber coverslip contacted the objective lens of an inverted microscope (Nikon TE200) via an immersion oil interface. A 60° C. fused silica dispersion prism was coupled optically to the back of the slide through a thin film of glycerol. Laser light was directed at the prism such that at the glass/sample interface it subtends an angle of approximately 68° C. to the normal of the slide and subsequently undergoes Total Internal Reflection (TIR). The critical angle for glass/water interface is 66°.

Fluorescence from single molecules of DNA-Cy3, produced by excitation with the surface-specific evanescent wave following TIR, was collected by the objective lens of the microscope and imaged onto an Intensified Charge Coupled Device (ICCD) camera (Pentamax, Princeton Instruments, N.J.). The image was recorded using a 532 nm excitation (frequency-doubled solid-state Nd.YAG, Antares, Coherent) with a 580 nm fluorescence (580DF30, Omega Optics, USA) filter for Cy3. Images were recorded with an exposure time of 500 ms at the maximum gain of 10 an the ICCD. Laser powers incident at the prism were 50 mW at 532 nm.

Single molecules were identified as described in Example 2.

The surface density of single molecules of DNA-Cy3 was measured at approximately 500 per 100 μm×100 μm image or 5×10^6 cm^2.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 7

<210> SEQ ID NO 1
<211> LENGTH: 13
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(13)
<223> OTHER INFORMATION: Modified base. n = 5'-(propargylamino)uridine

<400> SEQUENCE: 1

tcgcagccgn cca 13

<210> SEQ ID NO 2
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(21)
<223> OTHER INFORMATION: Modified base. n = 5-methyl cytosine with a TMR group coupled via a linker to the n4 position.

<400> SEQUENCE: 2

aaccctatgg acggctgcga n 21

<210> SEQ ID NO 3
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(21)
<223> OTHER INFORMATION: Modified base. n = methyl cytosine.

<400> SEQUENCE: 3

ntcgcagccg tccatagggt t 21

<210> SEQ ID NO 4
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: Description of Artificial Sequence:
Oligonucleotide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(40)
<223> OTHER INFORMATION: Modified base. N = (C6-amino)adenine

<400> SEQUENCE: 4

nctcaaccaa cctgccgacg ctccgagctg caagctactg 40

<210> SEQ ID NO 5
<211> LENGTH: 51
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Oligonucleotide

<400> SEQUENCE: 5

tcgactgctg acagtagctt gcagctcgga gcgtcggcag gttggttgag t 51

<210> SEQ ID NO 6
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Oligonucleotide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: Modified base. N = cytosine with a fluorescent
Cy3 group attached. M = thymine with hexaethylene
glycol attached.

<400> SEQUENCE: 6

ctgctgaagc gtcggcaggt 20

<210> SEQ ID NO 7
<211> LENGTH: 13
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Oligonucleotide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(13)
<223> OTHER INFORMATION: Modified base. N = adenine with hexaethylene
glycol attached.

<400> SEQUENCE: 7

acctgccgac gct 13

What is claimed is:

1. A method for analysing genome wide variation in an individual comprising:

i) randomly fragmenting a genome of said individual;

ii) generating sequence reads of multiple bases on all fragments of said genome;

iii) aligning the sequence reads with a known genomic reference sequence; and

iv) analysing variations between the sequence reads derived from the genome of the individual and the known genomic reference sequence.

2. A method according to claim **1** further comprising identifying regions of variation across the whole of the genome of said individual with respect to said reference sequence to provide a genetic signature for said individual.

3. A method according to claim **1**, wherein said fragments of genomic DNA are immobilised onto the surface of a solid support to form an array of polynucleotide molecules capable of interrogation and which molecules can be individually resolved by optical microscopy.

4. A method according to claim **1**, wherein each molecule is immobilised by covalent attachment to the surface of said solid support other than at that part of each molecule that can be interrogated.

5. A method according to claim **4**, wherein the sequences of said fragments are established by contacting the array

with each of the bases A, T G and C under conditions that permit the polymerase reaction to proceed and thereby form sequences complementary to those in the array, and determining the incorporation of a base at each of selected positions in the complementary sequences.

6. A method according to claim **5**, wherein the determination of the incorporation of said base is repeated between 10 and 20 times to generate a partial complementary sequence of between 10–20 nucleotides.

7. A method for analysing genome wide variation in an individual comprising:

i) randomly fragmenting a genome of said individual;

ii) arraying the fragments such that different fragments can be individually resolved by optical microscopy;

iii) generating sequence reads of multiple bases on all fragments of said genome;

iv) aligning said sequence reads with a known genomic reference sequence; and

v) analysing variations between the sequence reads derived from the individual sample and the known genomic reference sequence.

8. A method of generating a genetic signature for an individual, which method comprises:

i) immobilising fragments of the genome of said individual onto the surface of a solid support to form an array of polynucleotide molecules capable of interrogation, wherein the array allows the molecules to be individually resolved by optical microscopy, and wherein each molecule is immobilised by covalent bonding to the surface, other than at that part of each molecule that can be interrogated;

ii) contacting the fragments with a series of nucleotide primers comprising sequences capable of hybridising to regions on said fragments specific for each of the regions of variation identified according to claim **2**, in the presence of bases A, T, G and C under conditions that permit the polymerase reaction to proceed;

iii) determining the successive incorporation of a base at each of selected positions to produce sequences of a defined length complementary to the immobilised fragments;

iv) identifying the sequence of those fragments that have undergone the polymerase reaction to produce a genetic signature for said individual.

9. A method according to claim **7** or **8**, wherein in step iii) the incorporation of said base is repeated between 10 and 20 times to produce a complementary sequence of between 10 and 20 nucleotides.

* * * * *

EXHIBIT 7

US007598035B2

(12) **United States Patent**
Macevicz

(10) **Patent No.: US 7,598,035 B2**
(45) **Date of Patent: *Oct. 6, 2009**

(54) **METHOD AND COMPOSITIONS FOR ORDERING RESTRICTION FRAGMENTS**

(75) Inventor: **Stephen C. Macevicz**, Cupertino, CA (US)

(73) Assignee: **Solexa, Inc.**, Hayward, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 447 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **10/962,337**

(22) Filed: **Oct. 8, 2004**

(65) **Prior Publication Data**

US 2005/0042673 A1 Feb. 24, 2005

Related U.S. Application Data

(62) Division of application No. 10/706,118, filed on Nov. 12, 2003, now abandoned, which is a division of application No. 09/549,748, filed on Apr. 14, 2000, now Pat. No. 6,720,179, which is a division of application No. 09/028,128, filed on Feb. 23, 1998, now Pat. No. 6,054,276.

(51) **Int. Cl.**

C12Q 1/68	(2006.01)
C12P 19/34	(2006.01)
C07H 21/02	(2006.01)
C07H 21/04	(2006.01)
C07H 21/00	(2006.01)

(52) **U.S. Cl.** **435/6**; 435/91.1; 536/23.1; 536/24.3; 536/24.33; 536/25.3

(58) **Field of Classification Search** 435/6, 435/91.1, 91.2, 183; 436/94; 536/23.1, 24.3, 536/24.33, 25.3

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,293,652	A	10/1981	Cohen	
5,102,785	A	4/1992	Livak et al.	
5,196,328	A	3/1993	Tartof	
5,409,897	A *	4/1995	Thomas et al.	514/12
5,508,169	A	4/1996	Deugau	
5,604,097	A	2/1997	Brenner	
5,658,736	A	8/1997	Wong	
5,667,970	A	9/1997	Zhang	
5,695,937	A	12/1997	Kinzler	
5,710,000	A	1/1998	Sapolsky	
5,728,524	A	3/1998	Sibson	
5,804,382	A	9/1998	Sytkowski et al.	
5,834,201	A	11/1998	Hartley	
5,846,719	A	12/1998	Brenner et al.	
5,861,252	A	1/1999	Kambara et al.	
5,968,784	A	10/1999	Spinella et al.	
6,054,276	A *	4/2000	Macevicz	435/6
6,136,537	A *	10/2000	Macevicz	435/6

FOREIGN PATENT DOCUMENTS

EP	0 593 095 A1	4/1994
EP	0 761 822 A2	3/1997
WO	WO96/17082	6/1996
WO	WO98/10095	3/1998
WO	WO98/31838	7/1998

OTHER PUBLICATIONS

Chen et al., "Ordered shotgun sequencing, a strategy for integrated mapping and sequencing of YAC clones," *Genomics*, 17:651-656 (1993).

Evans, "Combinatoric strategies for genome mapping," *BioEssays*, 13:39-44 (1991).

Green et al., "Systematic generation of sequence-tagged sites for physical mapping of human chromosomes: application to the mapping of human chromosome 7 using yeast artificial chromosomes," *Genomics*, 11:548-564 (1991).

Hasan et al., "An Mbo II/Fok I trimming plasmid allowing consecutive cycles of precise 1- to 12-base-pair deletions in cloned DNA," *Gene*, 82:305-311 (1989).

Hasan et al., "A novel multistep method for generating precise unidirectional deletions using Bsp MI, a class-IIS restriction enzyme," *Gene*, 50:55-62 (1986).

Hudson et al., "An STS-based map of the human genome," *Science*, 270:1945-1954 (1995).

Kato, "RNA fingerprinting by molecular indexing," *Nucleic Acids Research*, 24:394-395 (1996).

Kato, "Description of the entire mRNA population by a 3' end cDNA fragment generated by class IIs restriction enzymes," *Nucleic Acids Research*, 23:3685-3690 (1995).

Michiels et al., "Molecular approaches to genome analysis: a strategy for the construction of ordered overlapping clone libraries," *CABIOS*, 3: 203-210 (1987).

Olson et al., "Random-clone strategy for genomic restriction mapping in yeast," *Proc.-Natl. Acad. Sci.*, 83:7826-7830 (1986).

Poustka and Lehrach, "Jumping libraries and linking libraries: the next generation of molecular tools in mammalian genetics," *Trends in Genetics*, 2:174-179 (1986).

Poustka and Lehrach, "Chromosome jumping: a long range cloning technique," in *Genetic Engineering: Principles and Methods*, J.K. Setlow, Editor, 10:169-193 (1988).

(Continued)

Primary Examiner—Frank W Lu
(74) *Attorney, Agent, or Firm*—Lee Ann Gorthey; King & Spalding LLP

(57) **ABSTRACT**

The invention provides a method for constructing a high resolution physical map of a polynucleotide. In accordance with the invention, nucleotide sequences are determined at the ends of restriction fragments produced by a plurality of digestions with a plurality of combinations of restriction endonucleases so that a pair of nucleotide sequences is obtained for each restriction fragment. A physical map of the polynucleotide is constructed by ordering the pairs of sequences by matching the identical sequences among the pairs.

5 Claims, 4 Drawing Sheets

OTHER PUBLICATIONS

Roach et al., "Pairwise end sequencing: A unified approach to genomic mapping and sequencing," *Genomics*, 26:345-353 (1995).
Sapolsky et al., "Mapping genomic library clones using oligonucleotide arrays," *Genomics*, 33:445-456 (1996).
Smith et al., "Genomic sequence sampling: a strategy for high resolution sequence-based physical mapping of complex genomes," *Nature Genetics*, 7:40-47 (1994).
Smith et al., "A simple method for DNA restriction site mapping," *Nucleic Acids Research*, 3: 2387-2398 (1976).
Velculescu et al., "Serial analysis of gene expression," *Science*, 270:484-487 (1995).
Wong et al., "Multiple-complete-digest restriction fragment mapping: Generating sequence-ready maps for large-scale DNA sequencing," *Proc. Natl. Acad. Sci*., 94:5225-5230 (1997).
Yi et al., "Construction of restriction fragment maps of 50- to 100-kilobase DNA", *Methods in Enzymology*, 218:651-671 (1993).
New England Biolabs 96/97 Catalog, pp. 15, 18, 22, 23 and 35. Published by New England Biolabs. Inc., 32 Tozer Road, Beverly, MA 01915-5599.
New England Biolabs 96/97 Catalog, pp. 36 and 50. Published by New England Biolabs. Inc., 32 Tozer Road, Beverly, MA 01915-5599.
New England Biolabs 96/97 Catalog, pp. 19, 37, 108 and 109. Published by New England Biolabs. Inc., 32 Tozer Road, Beverly, MA 01915-5599.
New England Biolabs 96/97 Catalog, p. 115. Published by New England Biolabs. Inc., 32 Tozer Road, Beverly, MA 01915-5599.
Howard et al., *Biotechniques*, 7:940-942 (1989).

* cited by examiner

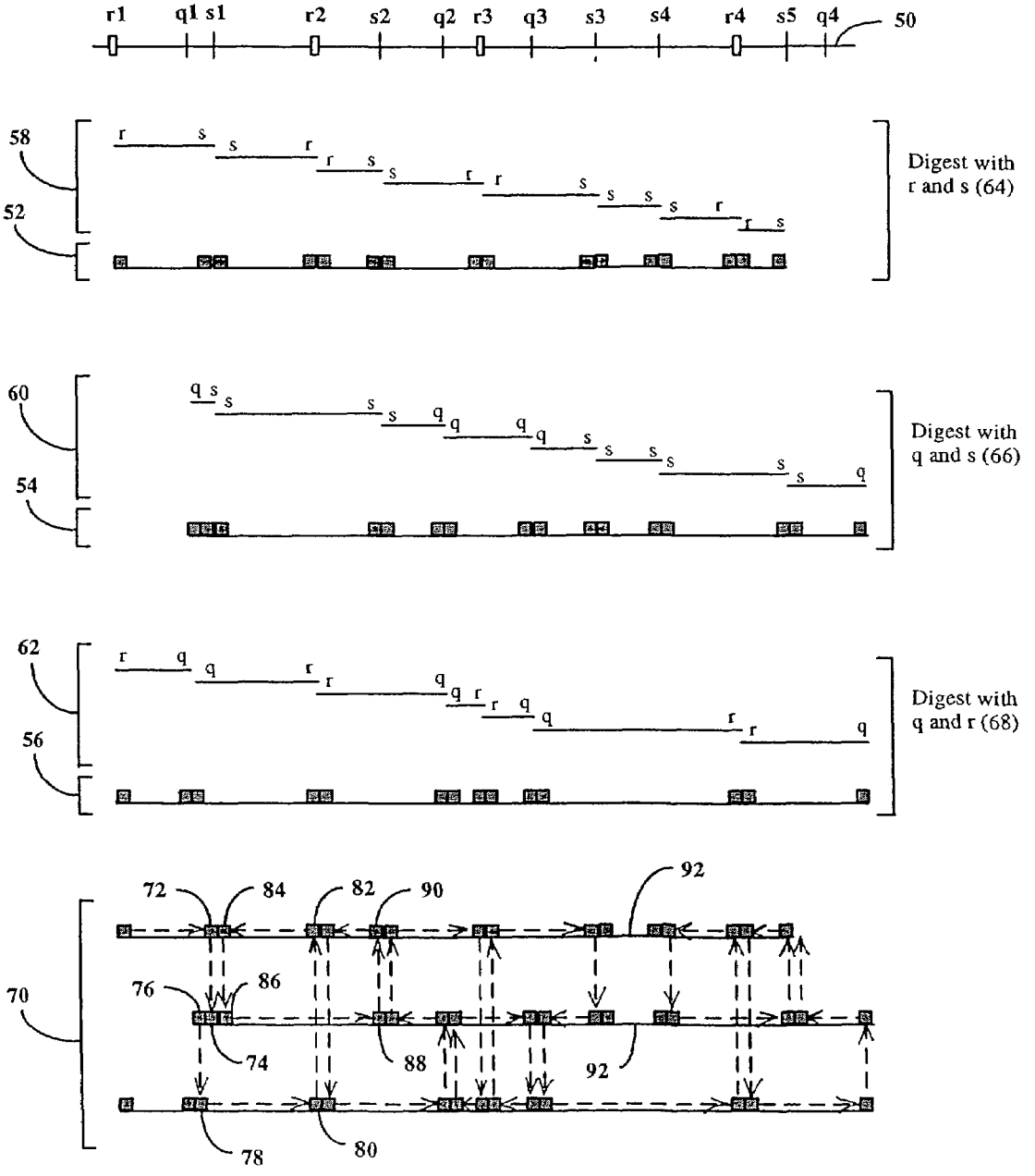

r1
q1 s1
r2
s2
q2
r3
q3
s3
s4
r4
s5
q4
50
58
52
Digest with r and s (64)
60
54
Digest with q and s (66)
62
56
Digest with q and r (68)
70
72
84
82
90
92
76
86
74
88
92
78
80

Fig. 1

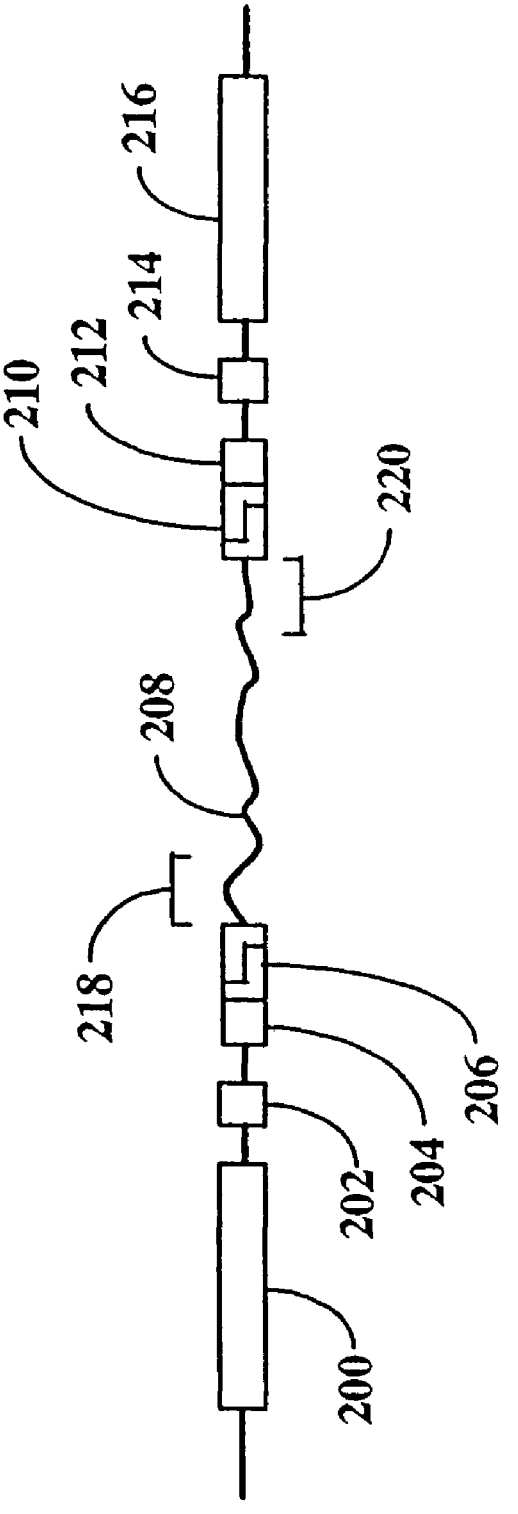


Fig. 2

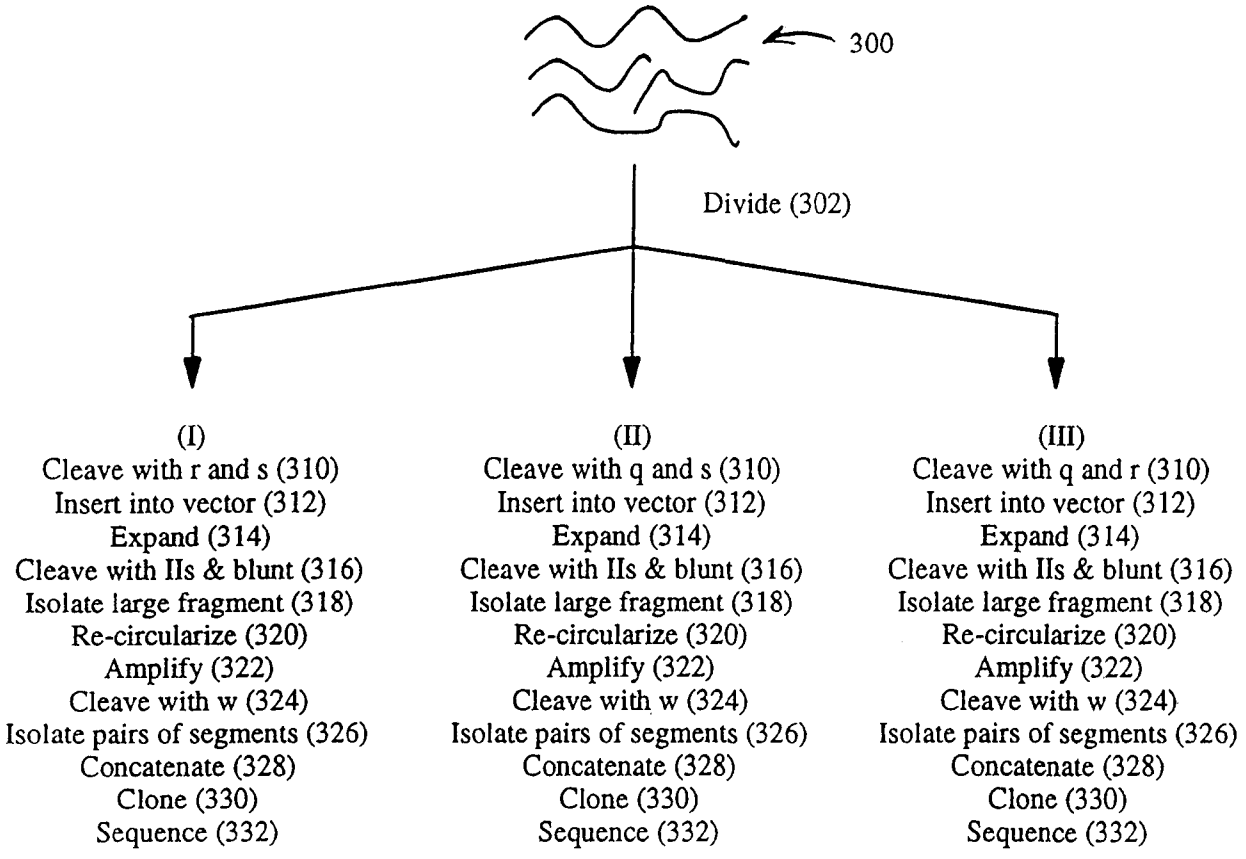

300
Divide (302)
(I)
Cleave with r and s (310)
Insert into vector (312)
Expand (314)
Cleave with IIs & blunt (316)
Isolate large fragment (318)
Re-circularize (320)
Amplify (322)
Cleave with w (324)
Isolate pairs of segments (326)
Concatenate (328)
Clone (330)
Sequence (332)
(II)
Cleave with q and s (310)
Insert into vector (312)
Expand (314)
Cleave with IIs & blunt (316)
Isolate large fragment (318)
Re-circularize (320)
Amplify (322)
Cleave with w (324)
Isolate pairs of segments (326)
Concatenate (328)
Clone (330)
Sequence (332)
(III)
Cleave with q and r (310)
Insert into vector (312)
Expand (314)
Cleave with IIs & blunt (316)
Isolate large fragment (318)
Re-circularize (320)
Amplify (322)
Cleave with w (324)
Isolate pairs of segments (326)
Concatenate (328)
Clone (330)
Sequence (332)

Fig. 3

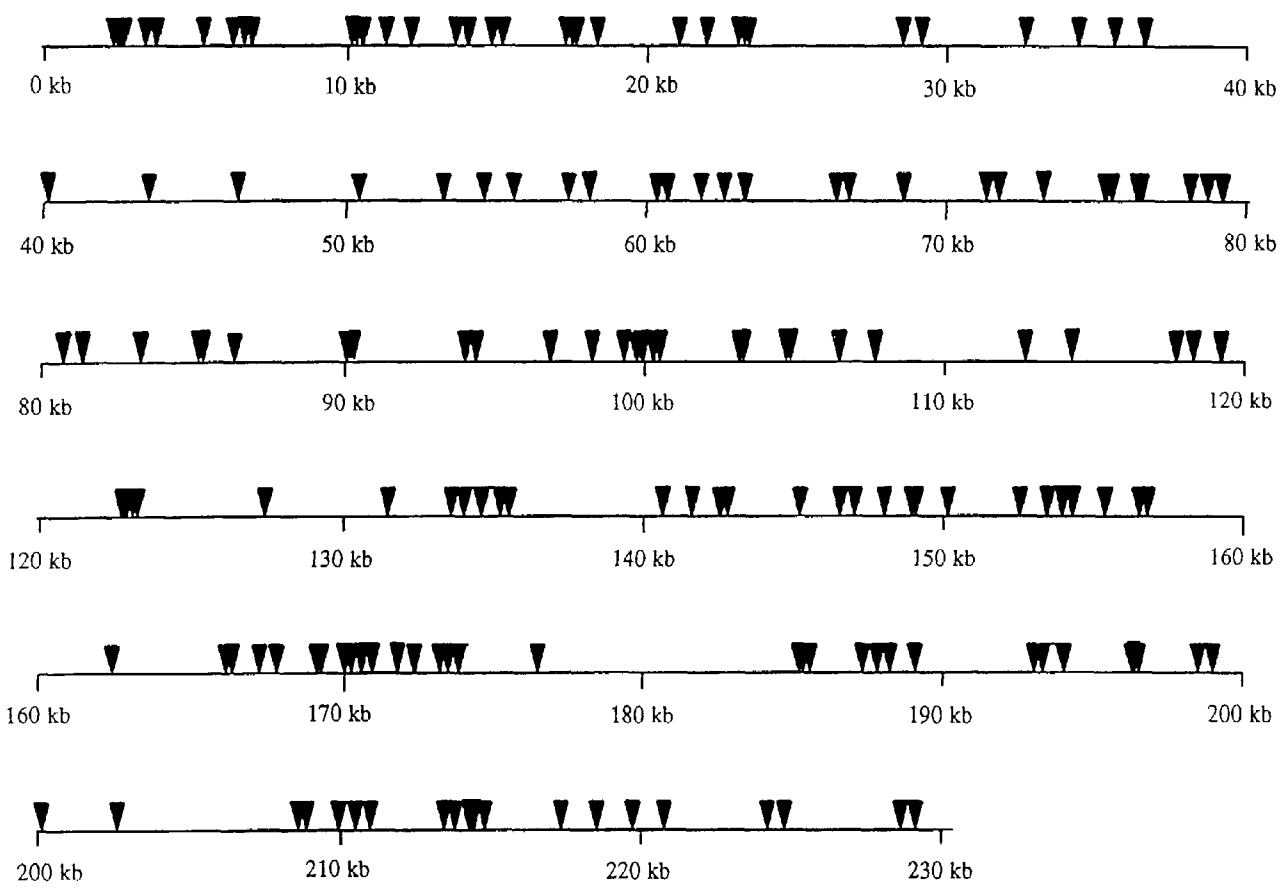

0 kb
10 kb
20 kb
30 kb
40 kb
40 kb
50 kb
60 kb
70 kb
80 kb
80 kb
90 kb
100 kb
110 kb
120 kb
120 kb
130 kb
140 kb
150 kb
160 kb
160 kb
170 kb
180 kb
190 kb
200 kb
200 kb
210 kb
220 kb
230 kb

Fig. 4

METHOD AND COMPOSITIONS FOR ORDERING RESTRICTION FRAGMENTS

This application is a divisional of application Ser. No. 10/706,118 filed on Nov. 12, 2003, now abandoned, which is a divisional of application Ser. No. 09/549,748 filed on Apr. 14, 2000, now U.S. Pat. No. 6,720,179, which is a divisional of application Ser. No. 09/028,128 filed on Feb. 23, 1998, now U.S. Pat. No. 6,054,276, all of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The invention relates generally to methods for construction physical maps of DNA, especially genomic DNA, and more particularly, to a method of providing high resolution physical maps by sequence analysis of concatenations of segments of restriction fragment ends.

BACKGROUND

Physical maps of one or more large pieces of DNA, such as a genome or chromosome, consist of an ordered collection of molecular landmarks that may be used to position, or map, a smaller fragment, such as clone containing a gene of interest, within the larger structure, e.g. U.S. Department of Energy, "Primer on Molecular Genetics," from Human Genome 1991-92 Program Report; and Los Alamos Science, 20: 112-122 (1992). An important goal of the Human Genome Project has been to provide a series of genetic and physical maps of the human genome with increasing resolution, i.e. with reduced distances in basepairs between molecular landmarks, e.g. Murray et al, Science, 265: 2049-2054 (1994); Hudson et al, Science, 270: 1945-1954 (1995); Schuler et al, Science, 274: 540-546 (1996); and so on. Such maps have great value not only in furthering our understanding of genome organization, but also as tools for helping to fill contig gaps in large-scale sequencing projects and as tools for helping to isolate disease-related genes in positional cloning projects, e.g. Rowen et al, pages 167-174, in Adams et al, editors, Automated DNA Sequencing and Analysis (Academic Press, New York, 1994); Collins, Nature Genetics, 9: 347-350 (1995); Rossiter and Caskey, Annals of Surgical Oncology, 2: 14-25 (1995); and Schuler et al (cited above). In both cases, the ability to rapidly construct high-resolution physical maps of large pieces of genomic DNA is highly desirable.

Two important approaches to genomic mapping include the identification and use of sequence tagged sites (STS's), e.g. Olson et al, Science, 245: 1434-1435 (1989); and Green et al, PCR Methods and Applications, 1: 77-90 (1991), and the construction and use of jumping and linking libraries, e.g. Collins et al, Proc. Natl. Acad. Sci., 81: 6812-6816 (1984); and Poustka and Lehrach, Trends in Genetics, 2: 174-179 (1986). The former approach makes maps highly portable and convenient, as maps consist of ordered collections of nucleotide sequences that allow application without having to acquire scarce or specialized reagents and libraries. The latter approach provides a systematic means for identifying molecular landmarks spanning large genetic distances and for ordering such landmarks via hybridization assays with members of a linking library.

Unfortunately, these approaches to mapping genomic DNA are difficult and laborious to implement. It would be highly desirable if there was an approach for constructing physical maps that combined the systematic quality of the jumping and linking libraries with the convenience and portability of the STS approach.

SUMMARY OF THE INVENTION

Accordingly, an object of my invention is to provide methods and materials for constructing high resolution physical maps of genomic DNA.

Another object of my invention is to provide a method of ordering restriction fragments from multiple enzyme digests by aligning matching sequences of their ends.

Still another object of my invention is to provide a high resolution physical map of a target polynucleotide that permits directed sequencing of the target polynucleotide with the sequences of the map.

Another object of my invention is to provide vectors for excising ends of restriction fragments for concatenation and sequencing.

Still another object of my invent is to provide a method monitoring the expression of genes.

A further object of my invention is to provide physical maps of genomic DNA that consist of an ordered collection of nucleotide sequences spaced at an average distance of a few hundred to a few thousand bases.

My invention achieves these and other objects by providing methods and materials for determining the nucleotide sequences of both ends of restriction fragments obtained from multiple enzymatic digests of a target polynucleotide, such as a fragment of a genome, or chromosome, or an insert of a cosmid, BAC, YAC, or the like. In accordance with the invention, a polynucleotide is separately digested with different combinations of restriction endonucleases and the ends of the restriction fragments are sequenced so that pairs of sequences from each fragment are produced. A physical map of the polynucleotide is constructed by ordering the pairs of sequences by matching the identical sequences among such pairs resulting from all of the digestions.

In the preferred embodiment, a polynucleotide is mapped by the following steps: (a) providing a plurality of populations of restriction fragments, the restriction fragments of each population having ends defined by digesting the polynucleotide with a plurality of combinations of restriction endonucleases; (b) determining the nucleotide sequence of a portion of each end of each restriction fragment of each population so that a pair of nucleotide sequences is obtained for each restriction fragment of each population; and (c) ordering the pairs of nucleotide sequences by matching the nucleotide sequences between pairs to form a map of the polynucleotide.

Another aspect of the invention is the monitoring gene expression by providing pairs of segments excised from cDNAs. In this embodiment, segments from each end of each cDNA of a population of cDNAs are ligated together to form pairs, which serve to identify their associated cDNAs. Concatenations of such pairs are sequenced by conventional techniques to provide information on the relative frequencies of expression in the population.

The invention provides a means for generating a high density physical map of target polynucleotides based on the positions of the restriction sites of predetermined restriction endonucleases. Such physical maps provide many advantages, including a more efficient means for directed sequencing of large DNA fragments, the positioning of expression sequence tags and cDNA sequences on large genomic fragments, such as BAC library inserts, thereby making positional candidate mapping easier; and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** graphically illustrates the concept of a preferred embodiment of the invention.

FIG. **2** provides a diagram of a vector for forming pairs of nucleotide sequences in accordance with a preferred embodiment of the invention.

FIG. **3** illustrates a scheme for carrying out the steps of a preferred embodiment of the invention.

FIG. **4** illustrates locations on yeast chromosome 1 where sequence information is provided in a physical map based on digestions with Hind III, Eco RI, and Xba I in accordance with the invention.

DEFINITIONS

As used herein, the process of "mapping" a polynucleotide means providing a ordering, or series, of sequenced segments of the polynucleotide that correspond to the actual ordering of the segments in the polynucleotide. For example, the following set of five-base sequences is a map of the polynucleotide below (SEQ ID NO: 8), which has the ordered set of sequences making up the map underlined:

```
                (gggtc, ttatt, aacct, catta, ccgga)

GTTGGGTCAACAAATTACCTTATTGTAACCTTCGCATTAGCCGGAGCCT
```

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g. 3-4, to several tens of monomeric units, e.g. 40-60. Whenever an oligonucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, unless otherwise noted. Usually oligonucleotides comprise the four natural nucleotides; however, they may also comprise non-natural nucleotide analogs. It is clear to those skilled in the art when oligonucleotides having natural or non-natural nucleotides may be employed, e.g. where processing by enzymes is called for, usually oligonucleotides consisting of natural nucleotides are required.

"Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one other such that every nucleotide in each strand undergoes Watson-Crick basepairing with a nucleotide in the other strand. The term also comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, and the like, that may be employed. In reference to a triplex, the term means that the triplex consists of a perfectly matched duplex and a third strand in which every nucleotide undergoes Hoogsteen or reverse Hoogsteen association with a basepair of the perfectly matched duplex.

As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the only proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like.

As used herein, the term "complexity" in reference to a population of polynucleotides means the number of different species of polynucleotide present in the population.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, segments of nucleotides at each end of restriction fragments produced from multiple digestions of a polynucleotide are sequenced and used to arrange the fragments into a physical map. Such a physical map consists of an ordered collection of the nucleotide sequences of the segments immediately adjacent to the cleavage sites of the endonucleases used in the digestions. Preferably, after each digestion, segments are removed from the ends of each restriction fragment by cleavage with a type IIs restriction endonuclease. Excised segments from the same fragment are ligated together to form a pair of segments. Preferably, collections of such pairs are concatenated by ligation, cloned, and sequenced using conventional techniques.

The concept of the invention is illustrated in FIG. **1** for an embodiment which employs three restriction endonucleases: r, q, and s. Polynucleotide (**50**) has recognition sites (r_1, r_2, r_3, and r_4) for restriction endonucleases r, recognition sites (q_1 through q_4) for restriction endonuclease q, and recognition sites (s_1 through s_5) for restriction endonuclease s. In accordance with the preferred embodiment, polynucleotide (**50**) is separately digested with r and s, q and s, and r and q to produce three populations of restriction fragments (**58**), (**60**), and (**62**), respectively. Segments adjacent to the ends of each restriction fragment are sequenced to form sets of pairs (**52**), (**54**), and (**56**) of nucleotide sequences, which for sake of illustration are shown directly beneath their corresponding restriction fragments in the correct order. Pairs of sequences from all three sets are ordered by matching sequences between pairs as shown (**70**). A nucleotide sequence (**72**) from a first pair is matched with a sequence (**74**) of a second pair whose other sequence (**76**), in turn, is matched with a sequence (**78**) of a third pair. The matching continues, as (**80**) is matched with (**82**), (**84**) with (**86**), (**88**) with (**90**), and so on, until the maximum number of pairs are included. It is noted that some pairs (**92**) do not contribute to the map. These correspond to fragments having the same restriction site at both ends. In other word, they correspond to situations where there are two (or more) consecutive restriction sites of the same type without other sites in between, e.g. s_3 and s_4 in this example. Preferably, algorithms used for assembling a physical map from the pairs of sequences can eliminate pairs having identical sequences.

Generally, a plurality of enzymes is employed in each digestion. Preferably, at least three distinct recognition sites are used. This can be accomplished by using three or more restriction endonucleases, such as Hind III, Eco RI, and Xba I, which recognize different nucleotide sequences, or by using restriction endonucleases recognizing the same nucleotide sequence, but which have different methylation sensitivities. That is, it is understood that a different "recognition site" may be different solely by virtue of a different methylation state. Preferably, a set of at least three recognition endonucleases is employed in the method of the invention. From this set a plurality of combinations of restriction endonucleases is formed for separate digestion of a target polynucleotide. Preferably, the combinations are "n−1" combinations of the set. In other words, for a set of n restriction endonucleases, the preferred combinations are all the combinations of n−1 restriction endonucleases. For example, as

illustrated in FIG. **1** where a set of three restriction endonucleases (r, q, and s) are employed, the n−1 combinations are (r, q), (r, s), and (q, s). Likewise, if four restriction endonucleases (r, q, s, and w) are employed, the n−1 combinations are (r, q, s), (r, q, w), (r, s, w), and (q, s, w). It is readily seen that where a set of n restriction endonucleases are employed the plurality of n−1 combinations is n.

Preferably, the method of the invention is carried out using a vector, such as that illustrated in FIG. **2**. The vector is readily constructed from commercially available materials using conventional recombinant DNA techniques, e.g. as disclosed in Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989). Preferably, pUC-based plasmids, such as pUC19, or λ-based phages, such as λ ZAP Express (Stratagene Cloning Systems, La Jolla, Calif.), or like vectors are employed. Important features of the vector are recognition sites (**204**) and (**212**) for two type IIs restriction endonucleases that flank restriction fragment (**208**). For convenience, the two type IIs restriction enzymes are referred to herein as "IIs_1" and "IIs_2", respectively. IIs_1 and IIs_2 may be the same or different. Recognition sites (**204**) and (**212**) are oriented so that the cleavage sites of IIs_1 and IIs_2 are located in the interior of restriction fragment (**208**). In other words, taking the 5' direction as "upstream" and the 3' direction as "downstream," the cleavage site of IIs_1 is downstream of its recognition site and the cleavage site of IIs_2 is upstream of its recognition site. Thus, when the vector is cleaved with IIs_1 and IIs_2 two segments (**218**) and (**220**) of restriction fragment (**208**) remain attached to the vector. The vector is then re-circularized by ligating the two ends together, thereby forming a pair of segments. If such cleavage results in one or more single stranded overhangs, i.e. one or more non-blunt ends, then the ends are preferably rendered blunt prior to re-circularization, for example, by digesting the protruding strand with a nuclease such as Mung bean nuclease, or by extending a 3' recessed strand, if one is produced in the digestion. The ligation reaction for re-circularization is carried out under conditions that favor the formation of covalent circles rather than concatemers of the vector. Preferably, the vector concentration for the ligation is between about 0.4 and about 4.0 μg/ml of vector DNA, e.g. as disclosed in Collins et al, Proc. Natl. Acad. Sci., 81: 6812-6812 (1984), for α-based vectors. For vectors of different molecular weight, the concentration range is adjusted appropriately.

In the preferred embodiments, the number of nucleotides identified depends on the "reach" of the type IIs restriction endonucleases employed. "Reach" is the amount of separation between a recognition site of a type IIs restriction endonuclease and its cleavage site, e.g. Brenner, U.S. Pat. No. 5,559,675. The conventional measure of reach is given as a ratio of integers, such as "(16/14)", where the numerator is the number of nucleotides from the recognition site in the 5'→3' direction that cleavage of one strand occurs and the denominator is the number of nucleotides from the recognition site in the 3'→5' direction that cleavage of the other strand occurs. Preferred type IIs restriction endonucleases for use as IIs_1 and IIs_2 in the preferred embodiment include the following: Bbv I, Bce 83 I, Bcef I, Bpm I, Bsg I, BspLU II III, Bst 71 I, Eco 57 I, Fok I, Gsu I, Hga I, Mme I, and the like. In the preferred embodiment, a vector is selected which does not contain a recognition site, other than (**204**) and (**212**), for the type IIs enzyme(s) used to generate pairs of segments; otherwise, re-circularization cannot be carried out.

Preferably, a type IIs restriction endonuclease for generating pairs of segments has as great a reach as possible to maximize the probability that the nucleotide sequences of the segments are unique. This in turn maximizes the probability that a unique physical map can be assembled. If the target polynucleotide is a bacterial genome of 1 megabase, for a restriction endonuclease with a six basepair recognition site, about 250 fragments are generated (or about 500 ends) and the number of nucleotides determined could be as low as five or six, and still have a significant probability that each end sequence would be unique. Preferably, for polynucleotides less than or equal to 10 megabases, at least 8 nucleotides are determined in the regions adjacent to restriction sites, when a restriction endonuclease having a six basepair recognition site is employed. Generally for polynucleotides less than or equal to 10 megabases, 9-12 nucleotides are preferably determined to ensure that the end sequences are unique. In the preferred embodiment, type IIs enzymes having a (16/14) reach effectively provide 9 bases of unique sequence (since blunting reduces the number of bases to 14 and 5 bases are part of the recognition sites (**206**) or (**210**)). In a polynucleotide having a random sequence of nucleotides, a 9-mer appears on average about once every 262,000 bases. Thus, 9-mer sequences are quite suitable for uniquely labeling restriction fragments of a target polynucleotide corresponding to a typical yeast artificial chromosome (YACs) insert, i.e. 100-1000 kilobases, bacterial artificial chromosome (BAC) insert, i.e. 50-250 kilobases, and the like.

Immediately adjacent to IIs sites (**204**) and (**212**) are restriction sites (**206**) and (**210**), respectively that permit restriction fragment (**208**) to be inserted into the vector. That is, restriction site (**206**) is immediately downstream of (**204**) and (**210**) is immediately upstream of (**212**). Preferably, sites (**204**) and (**206**) are as close together as possible, even overlapping, provided type IIs site (**206**) is not destroyed upon cleavage with the enzymes for inserting restriction fragment (**208**). This is desirable because the recognition site of the restriction endonuclease used for generating the fragments occurs between the recognition site and cleavage site of type IIs enzyme used to remove a segment for sequencing, i.e. it occurs within the "reach" of the type IIs enzyme. Thus, the closer the recognition sites, the larger the piece of unique sequence can be removed from the fragment. The same of course holds for restriction sites (**210**) and (**212**). Preferably, whenever the vector employed is based on a pUC plasmid, restriction sites (**206**) and (**210**) are selected from either the restriction sites of polylinker region of the pUC plasmid or from the set of sites which do not appeal in the pUC. Such sites include Eco RI, Apo I, Ban II, Sac I, Kpn I, Acc65 I, Ava I, Xma I, Sma I, Bam HI, Xba I, Sal I, Hinc II, Acc I, BspMI, Pst I, Sse8387 I, Sph I, Hind III, Afl II, Age I, Bsp120 I, Asc I, Bbs I, Bcl I, Bgl II, Blp I, BsaA I, Bsa BI, Bse RI, Bsm I, Cla I, Bsp EI, BssH II, Bst BI, BstXI, Dra III, Eag I, Eco RV, Fse I, Hpa I, Mfe I, Nae I, Nco I, Nhe I, Not I, Nru I, Pac I, Xho I, Pme I, Sac II, Spe I, Stu I, and the like. Preferably, six-nucleotide recognition sites (i.e. "6-cutters") are used, and more preferably, 6-cutters leaving four-nucleotide protruding strands are used.

Preferably, the vectors contain primer binding sites (**200**) and (**216**) for primers p_1 and p_2, respectively, which may be used to amplify the pair of segments by PCR after re-circularization. Recognition sites (**202**) and (**214**) are for restriction endonucleases w_1 and w_2, which are used to cleave the pair of segments from the vector after amplification. Preferably, w_1 and w_2, which may be the same or different, are type IIs restriction endonucleases whose cleavage sites correspond to those of (**206**) and (**210**), thereby removing surplus, or non-informative, sequence (such as the recognition sites (**204**) and (**212**)) and generating protruding ends that permit concatenation of the pairs of segments.

FIG. **3** illustrates steps in a preferred method using vectors of FIG. **2**. Genomic or other DNA (**400**) is obtained using conventional techniques, e.g. Herrmann and Frischauf, Methods in Enzymology, 152: 180-183 (1987); Frischauf, Methods in Enzymology, 152: 183-199 (1987), or the like, after which it is divided (302) into aliquots that are separately

digested (**310**) with combinations restriction endonucleases, as shown in FIG. **3** for the n−1 combinations of the set of enzymes r, s, and q. Preferably, the resulting fragments are treated with a phosphatase to prevent ligation of the genomic fragments with one another before or during insertion into a vector. Restriction fragments are inserted (**312**) into vectors designed with cloning sites to specifically accept the fragments. That is, fragments digested with r and s are inserted into a vector that accepts r-s fragments. Fragments having the same ends, e.g. r-r and s-s, are not cloned since information derived from them does not contribute to the map. r-s fragments are of course inserted into the vector in both orientations. Thus, for a set of three restriction endonucleases, only three vectors are required, e.g. one each for accepting r-s, r-q, and s-q fragments. Likewise, for a set of four restriction endonucleases, e.g. r, s, q, and t, only six vectors are required, one each for accepting r-s, r-q, r-t, s-q, s-t, and q-t fragments.

After insertion, a suitable host is transformed with the vectors and cultured, i.e. expanded (**314**), using conventional techniques. Transformed host cells are then selected, e.g. by plating and picking colonies using a standard marker, e.g. β-glactosidase/X-gal. A large enough sample of transformed host cells is taken to ensure that every restriction fragment is present for analysis with a reasonably large probability. This is similar to the problem of ensuring representation of a clone of a rare mRNA in a cDNA library, as discussed in Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references. Briefly, the number of fragments, N, that must be in a sample to achieve a given probability, P, of including a given fragment is the following: $N=\ln(1-P)/\ln(1-f)$, where f is the frequency of the fragment in the population. Thus, for a population of 500 restriction fragments, a sample containing 3454 vectors will include at least one copy of each fragment (i.e. a complete set) with a probability of 99.9%; and a sample containing 2300 vectors will include at least one copy of each fragment with a probability of 99%. The table below provides the results of similar calculations for target polynucleotides of different sizes:

TABLE I

Size of Target Polynucleotide (basepairs)	Average fragment size after cleavage with 2 six-cutters (No. of fragments) [Sample size for complete set with 99% probability]	Average fragment size after cleavage with 3 six-cutters (No. of fragments) [Sample size for complete set with 99% probability]
2.5×10^5	2048 (124) [576]	1365 (250) [1050]
5×10^5	2048 (250) [1050]	1365 (500) [2300]
1×10^6	2048 (500) [2300]	1365 (1000) [4605]

After selection, the vector-containing hosts are combined and expanded in cultured. The vectors are then isolated, e.g. by a conventional mini-prep, or the like, and cleaved with IIs_1 and IIs_2 (**316**). The fragments comprising the vector and ends (i.e. segments) of the restriction fragment insert are isolated, e.g. by gel electrophoresis, blunted (**316**), and re-circularized (**320**). The resulting pairs of segments in the re-circularized vectors are then amplified (**322**), e.g. by polymerase chain reaction (PCR), after which the amplified pairs are cleaved with w (**324**) to free the pairs of segments, which are isolated (**326**), e.g. by gel electrophoresis. The isolated pairs are concatenated (**328**) in a conventional ligation reaction to produce concatemers of various sizes, which are separated, e.g. by gel electrophoresis. Concatemers greater than about 200-300 basepairs are isolated and cloned (**330**) into a standard sequencing vector, such as M13. The sequences of the cloned concatenated pairs are analyzed on a conventional DNA sequencer, such as a model 377 DNA sequencer from Perkin-Elmer Applied Biosystems Division (Foster City, Calif.).

In the above embodiment, the sequences of the pairs of segments are readily identified between sequences for the recognition site of the enzymes used in the digestions. For example, when pairs are concatenated from fragments of the r and s digestion after cleavage with a type IIs restriction endonuclease of reach (16/14), the following pattern is observed (SEQ ID NO: 1):

```
. . . NNNNrrrrrrNNNNNNNNNNNNNNNNNNqqqqqqNNNNN

N . . .
```

where "r" and "q" represent the nucleotides of the recognition sites of restriction endonuclease r and q, respectively, and where the N's are the nucleotides of the pairs of segments. Thus, the pairs are recognized by their length and their spacing between known recognition sites.

Pairs of segments are ordered by matching the sequences of segments between pairs. That is, a candidate map is built by selecting pairs that have one identical and one different sequence. The identical sequences are matched to form a candidate map, or ordering, as illustrated below for pairs (s_1, s_2), (s_3, s_2), (s_3, s_4), (s_5, s_4), and (s_5, s_6), where the "s_k's" represent the nucleotide sequences of the segments:

```
. . .  s1---- s2

s3---------------- s2

s3---------------------------------- --- s4

                                    s5------- s4

s5---------------- s6 . . .
```

Sequence matching and candidate map construction is readily carried out by computer algorithms, such as the Fortran code provided in Appendix A. Preferably, a map construction algorithm initially sorts the pairs to remove identical pairs prior to map construction. That is, preferably only one pair of each kind is used in the reconstruction. If for two pairs, (s_i, s_j) and (s_m, s_n), $s_i=s_m$ and $s_j=s_n$, then one of the two can be eliminated prior to map construction. As pointed out above, such additional pairs either correspond to restriction fragments such as (**92**) of FIG. **1** (no sites of a second or third restriction endonuclease in its interior) or they are additional copies of pairs (because of sampling) that can be used in the analysis. Preferably, an algorithm selects the largest candidate map as a solution, i.e. the candidate map that uses the maximal number of pairs.

The vector of FIG. **2** can also be used for determining the frequency of expression of particular cDNAs in a cDNA library. Preferably, cDNAs whose frequencies are to be determined are cloned into a vector by way of flanking restriction sites that correspond to those of (**206**) and (**210**). Thus, cDNAs may be cleaved from the library vectors and directionally inserted into the vector of FIG. **2**. After insertion, analysis is carried out as described for the mapping embodiment, except that a larger number of concatemers are sequenced in order to obtain a large enough sample of cDNAs for reliable data on frequencies.

Example 1

Constructing a Physical Map of Yeast Chromosome I with Hind III, Eco RI, and Xba I

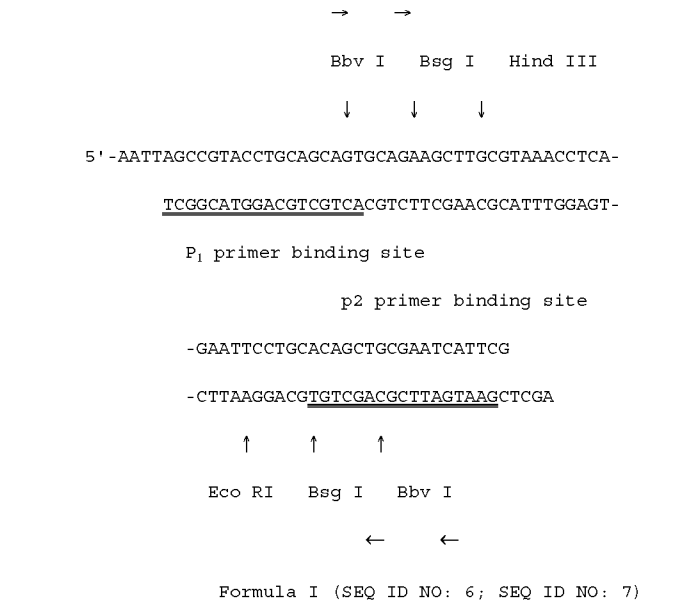
In this example, a physical map of the 230 kilobase yeast chromosome 1 is constructed using pUC19 plasmids modified in accordance with FIG. **2**. The chromosome is separately digested to completion with the following combinations of enzymes: Hind III and Eco RI, Hind III and Xba I, and Eco RI and Xba Ito generate three populations of restriction fragments. Fragments from each population are inserted into separate pUC19 plasmids, one for each restriction fragment having different ends. That is, restriction fragments from the Hind III-Eco RI digestion are present in three types, ones with a Hind III-digested end and an Eco RI-digested end ("H-E" fragments), one with only Hind III-digested ends ("H-H" fragments), and ones with only Eco RI-digested fragments ("E-E" fragments). Likewise, restriction fragments from the Hind III-Kba I digestion are present in three types, ones with a Hind III-digested end and an Xba I-digested end ("H-X" fragments), one with only Hind III-digested ends ("H-H" fragments), and ones with only Xba I-digested fragments ("X-X" fragments). Finally, restriction fragments from the Xba I-Leo RI digestion are present in three types, ones with a Xba I-digested end and an Leo RI-digested end ("X-E" fragments), one with only Xba I-digested ends ("X-X" fragments), and ones with only Leo RI-digested fragments ("E-E" fragments). Thus, the plasmid for the Hind III-Eco RI digestion accepts H-E fragments; the plasmid for the Hind III-Xba I digestion accepts H-X fragments; and the plasmid for the Xba I-Leo RI digestion accepts X-E fragments. The construction of the plasmid for accepting H-E fragments is described below. The other plasmids are constructed in a similar manner. Synthetic oligonucleotides (i) through (iv) are combined with a Eco RI- and Hind III-digested pUC19 in a ligation reaction so that they assemble into the double stranded insert of Formula I.

(i) 5'-AATTAGCCGTACCTGCAGCAGTGCAGAAGCTTGCGT (SEQ ID NO: 2)

(ii) 5'-AAACCTCAGAATTCCTGCACAGCTGCGAATCATTCG (SEQ ID NO: 3)

(iii) 5'-AGCTCGAATGATTCGCAGCTGTGCAGGAATTCTGAG (SEQ ID NO: 4)

(iv) 5'-GTTTACGCAAGCTTCTGCACTGCTGCAGGTACGGCT (SEQ ID NO: 5)



Formula I (SEQ ID NO: 6; SEQ ID NO: 7)

Note that the insert has compatible ends to the Eco RI-Hind III-digested plasmid, but that the original Eco RI and Hind III sites are destroyed upon ligation. The horizontal arrows above and below the Bsg I and Bbv I sites indicate the direction of the cleavage site relative to the recognition site of the enzymes. After ligation, transformation of a suitable host, and expansion, the modified pUC19 is isolated and the insert is sequenced to confirm its identity.

Yeast chromosome 1 DNA is separated into three aliquots of about 5 μg DNA (0.033 pmol) each, which are then separately digested to completion with Hind III and Eco RI, Hind III and Xba I, and Eco RI and Xba I, respectively. For each of the three populations, the same procedure is followed, which is described as follows for the pUC19 designed for H-E fragments.

Since each enzyme recognizes a six basepair recognition sequence, about 100-140 fragments are produced for a total of about 3.3 pmol of fragments, about fifty percent of which are H-E fragments. 5.26 μg (3 pmol) of plasmid DNA is digested with Eco RI and Hind III in Eco RI buffer as recommended by the manufacturer (New England Biolabs, Beverly, Mass.), purified by phenol extraction and ethanol precipitation, and ligated to the H-E fragments of the mixture in a standard ligation reaction. A bacterial host is transformed, e.g. by electroporation, and plated so that hosts containing recombinant plasmids are identified by white colonies. The digestion of the yeast chromosome I generates about 124 fragments of the three types, about fifty percent of which are H-E fragments and about twenty-five percent each are H-H or E-E fragments. About 290 colonies are picked for H-E fragments, and about 145 each are picked for H-H and E-E fragments. The same procedure is carried out for all the other types of fragments, so that six populations of transformed hosts are obtained, one each for H-E, H-X, X-E, H-H, E-E, and X-X fragments. Each of the populations is treated separately as follows: About 10 μg of plasmid DNA is digested to completion with Bsg I using the manufacturer's protocol (New England Biolabs, Beverly, Mass.) and after phenol extraction the vector/segment-containing fragment is isolated, e.g. by gel electrophoresis. The ends of the isolated fragment are then blunted by Mung bean nuclease (using the manufacturer's recommended protocol, New England Biolabs), after which the blunted fragments are purified by phenol extraction and ethanol precipitation. The fragments are then resuspended in a ligation buffer at a concentration of about 0.05 μg/ml in 20 1-ml reaction volumes. The dilution is designed to promote self-ligation of the fragments, following the protocol of Collins et al, Proc. Natl. Acad. Sci., 81: 6812-6816 (1984). After ligation and concentration by ethanol precipitation, phages from the 20 reactions are combined. The pairs of segments carried by the plasmids are then amplified by PCR using primers p_1 and p_2. The amplified product is purified by phenol extraction and ethanol precipitation, after which it is cleaved with Bbv I using the manufacturer's recommended protocol (New England Biolabs). After isolation by polyacrylamide gel electrophoresis, the pairs are concatenated by carrying out a conventional ligation reaction. The concatenated fragments are then separated by polyacrylamide gel electrophoresis and concatemers greater than about 200 basepairs are isolated and ligated into an equimolar mixture of three Phagescript SK sequencing vectors (Stratagene Cloning Systems, La Jolla, Calif.), separately digested with Hind III, Eco RI, and Hind III and Eco RI, respectively. (Other appropriate mixtures and digestions are employed when different combinations of enzymes are used). Preferably, a number of clones are expanded and sequenced that ensure with a probability of at least 99% that all of the pairs of the aliquot are sequenced. A "lane" of sequence data (about 600 bases) obtained with conventional sequencing provides the sequences of about 25 pairs of segments. Thus, after transfection, about 13 individual clones are expanded and sequenced on a commercially available DNA sequencer, e.g. PE Applied Biosystems model 377, to give the identities of about 325 pairs of segments. The other sets of fragments require an additional 26 lanes of sequencing (13 each for the H-X and X-E fragments).

FIG. **4** illustrates the positions on yeast chromosome 1 of pairs of segments ordered in accordance with the algorithm of Appendix A. The relative spacing of the segments along the chromosome is only provided to show the distribution of sequence information along the chromosome.

Example 2

Directed Sequencing of Yeast Chromosome 1 Using Restriction Map Sequences as Spaced PCR Primers

In this example, the 14-mer segments making up the physical map of Example 1 are used to separately amplify by PCR fragments that collectively cover yeast 1 chromosome. The PCR products are inserted into standard M13mp19, or like, sequencing vectors and sequenced in both the forward and reverse directions using conventional protocols. For fragments greater than about 800 basepairs, the sequence information obtained in the first round of sequencing is used to synthesized new sets of primers for the next round of sequencing. Such directed sequencing continues until each fragment is completely sequenced. Based on the map of Example 1, 174 primers are synthesized for 173 PCRs. The total number of sequencing reactions required to cover yeast chromosome 1 depends on the distribution of fragment sizes, and particularly, how many rounds of sequencing are required to cover each fragment: the larger the fragment, the more rounds of sequencing that are required for full coverage. Full coverage of a fragment is obtained when inspection of the sequence information shows that complementary sequences are being identified. Below, it is assumed that conventional sequencing will produce about 400 bases at each end of a fragment in each round. Inspection shows that the distribution of fragment sizes from the Example 1 map of yeast chromosome I are shown below together with reaction and primer requirements:

Round of Sequencing	Fragment size range	Number of Fragments	Number of Seq. or PCR Primers	Number of Sequencing Reactions
1	>0	174	174	348
2	>800	92	184	184
3	>1600	53	106	106
4	>2400	28	56	56
5	>3200	16	32	32

-continued

Round of Sequencing	Fragment size range	Number of Fragments	Number of Seq. or PCR Primers	Number of Sequencing Reactions
6	>4000	7	14	14
7	>4800	5	10	10
8	>5600	1	2	2
		Total No. of Primers:	578	752
			Seq. reactions for map:	39
			Total No. of Reactions:	791

This compares to about 2500-3000 sequencing reactions that are required for full coverage using shotgun sequencing.

APPENDIX A

Computer Code for Ordering Pairs into a Physical Map

```
      program opsort
c
c     opsort reads ordered pairs from disk files
c     p1.dat, p2.dat, and p3.dat. and sorts
c     them into a physical map.
c
      character*1 op(1000,2,14),w(14),x(14)
      character*1 fp(1000,2,14),test(14)
c
c
      open(1,file='p1.dat',status='old')
      open(5,file='olist.dat',status='replace')
c
c
      nop=0
      read(1,100)nop1
      nop=nop + nop1
      do 101 j=1,nop
        read(1,102)(w(i),i=1,14),
     +             (x(k),k=1,14)
      do 121 kk=1,14
        op(j,1,kk)=w(kk)
        op(j,2,kk)=x(kk)
121     continue
101   continue
      read(1,100)nop2
      nop=nop + nop2
      do 1011 j=nop1+1,nop
        read(1,102)(w(i),i=1,14),
     +             (x(k),k=1,14)
      do 1211 kk=1,14
        op(j,1,kk)=w(kk)
        op(j,2,kk)=x(kk)
1211    continue
1011  continue
c
      close(1)
c
      write(5,110)nop1,nop2,nop
110   format(3(2x,i4))
c
c
      open(1,file='p2.dat',status='old')
      read(1,100)nop3
      nop=nop + nop3
      do 104 j=nop1+nop2+1,nop
        read(1,102)(w(i),i=1,14),
     +             (x(k),k=1,14)
      do 122 kk=1,14
        op(j,1,kk)=w(kk)
        op(j,2,kk)=x(kk)
122     continue
104   continue
c
      read(1,100)nop4
      nop=nop + nop4
```

APPENDIX A-continued

Computer Code for Ordering Pairs into a Physical Map

```
      do 1041 j=nop1+nop2+nop3+1,nop
        read(1,102)(w(i),i=1,14),
     +             (x(k),k=1,14)
      do 1221 kk=1,14
        op(j,1,kk)=w(kk)
        op(j,2,kk)=x(kk)
1221    continue
1041  continue
c
      close(1)
      write(5,1108)nop1,nop2,nop3,nop4,nop
1108  format(5(2x,i4))
c
c
      open(1,file='p3.dat',status='old')
      read(1,100)nop5
      nop=nop + nop5
      do 105 j=nop1+nop2+nop3+nop4+1,nop
        read(1,102)(w(i),i=1,14),
     +             (x(k),k=1,14)
      do 123 kk=1,14
        op(j,1,kk)=w(kk)
        op(j,2,kk)=x(kk)
123     continue
105   continue
c
      read(1,100)nop6
      nop=nop + nop6
      do 1051 j=nop1+nop2+nop3+nop4+nop5+1,nop
        read(1,102)(w(i),i=1,14),
     +             (x(k),k=1,14)
      do 1231 kk=1,14
        op(j,1,kk)=w(kk)
        op(j,2,kk)=x(kk)
1231    continue
1051  continue
c
      close(1)
      write(5,1109)nop1,nop2,nop3,nop4,nop5,nop6,nop
1109  format(7(2x,i4))
c
c
100   format(i4)
102   format(2(2x,14a1))
111   format(/)
c
c
      write(5,111)
      do 120 m=1,nop
        write(5,102)(op(m,1,i),i=1,14),
     +              (op(m,2,k),k=1,14)
        write(*,102)(op(m,1,i),i=1,14),
     +              (op(m,2,k),k=1,14)
120     continue
c
c
      write(5,111)
      do 1100 i=1,14
```

APPENDIX A-continued

Computer Code for Ordering Pairs into a Physical Map

```
            test(i)=op(1,2,i)
                fp(1,1,i)=op(1,1,i)
                fp(1,2,i)=op(1,2,i)
1100        continue
c
          nxx=nop
          ns=1
c
1000      continue
          ne=0
          do 2000 ix=2,nxx
            nt=0
            do 2100 jx=1,14
            if(test(jx).ne.op(ix,1,jx)) then
              nt=nt+1
              endif
2100      continue
            if(nt.eq.0) then
              ns=ns+1
c
              ne=ne+1
              if(ne.gt.1) then
                write(*,1003)
1003      format(1x,'ne is gt 1')
              endif
c
              do 2200 kx=1,14
                fp(ns,1,kx)=op(ix,1,kx)
                fp(ns,2,kx)=op(ix,2,kx)
                test(kx)=op(ix,2,kx)
2200            continue
                  mm=0
```

APPENDIX A-continued

Computer Code for Ordering Pairs into a Physical Map

```
                do 2300 mx=1,nxx
                  if(mx.eq.ix) then
                    goto 2300
                  else
                    mm=mm+1
                    do 2400 ma=1,14
                    op(mm,1,ma)=op(mx,1,ma)
                    op(mm,2,ma)=op(mx,2,ma)
2400                continue
                  endif
2300              continue
                endif
2000          continue
              nxx=nxx-1
              if(ne.ne.0) then
                goto 1000
                endif
c
c
          do 1220 m=1,ns
            write(5,102)(fp(m,1,i),i=1,14),
     +                  (fp(m,2,k),k=1,14)
            write(*,102)(fp(m,1,i),i=1,14),
     +                  (fp(m,2,k),k=1,14)
1220        continue
          write(*,100)ns
c
          close(5)
c
          end
```

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 6

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 40

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AATTAGCCGT ACCTGCAGCA GTGCAGAAGC TTGCGT 36

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 nucleotides
(B) TYPE: nucleic acid

-continued

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AAACCTCAGA ATTCCTGCAC AGCTGCGAAT CATTCG 36

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGCTCGAATG ATTCGCAGCT GTGCAGGAAT TCTGAG 36

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTTTACGCAA GCTTCTGCAC TGCTGCAGGT ACGGCT 36

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AATTAGCCGT ACCTGCAGCA GTGCAGAAGC TTGCGTAAAC CTCAGAATTC 50

CTGCACAGCT GCGAATCATT CG 72

I claim:

1. A method of forming a linked pair of segments from each fragment of a population of polynucleotide fragments, wherein each said fragment is in a vector, said vector comprises two type IIs restriction endonuclease recognition sites, one of said type IIs restriction endonuclease recognition sites is adjacent to one end of the fragment and another of said type IIs restriction endonuclease recognition sites is adjacent to another end of the fragment, and one or more type IIs restriction endonucleases recognizing said type IIs restriction endonuclease recognition sites have two cleavage sites within the interior of the fragment, the method comprising:

treating each said vector and fragment with said one or more type IIs restriction endonucleases recognizing said type IIs restriction recognition sites, such that each fragment is cleaved at said cleavage sites, thereby removing the region of the fragment between said cleavage sites, whereby a pair of segments from said fragment remain connected to each other via said vector after said removing and each of said segments has a cleaved end; and

ligating together the cleaved ends produced by said removing thereby forming a linked pair of segments from each said fragment.

2. The method of claim **1** wherein the two type IIs recognition sites are recognized by the same type IIs restriction endonuclease.

3. A method of forming linearized vectors having pairs of segments from a population of polynucleotide fragments, the method comprising the steps of:

i) inserting each of said polynucleotide fragments from said population into a vector having two type IIs restriction endonuclease recognition sites and forming vectors containing said polynucleotide fragments such that each of the vectors containing said polynucleotide fragments comprises a fragment from said population of polynucleotide fragments, one of said type IIs restriction endonuclease recognition sites in each of the vectors containing said polynucleotide fragments is adjacent to one end of the fragment and another of said type IIs endonuclease recognition sites in each of the vectors containing said polynucleotide fragments is adjacent to another end of the fragment, and one or more type IIs restriction endonucleases recognizing said type IIs restriction endonuclease recognition sites have two cleavage sites within the interior of the fragment; and

ii) treating each of the vectors containing said polynucleotide fragments with said one or more type IIs restriction

endonucleases recognizing the type IIs restriction endonuclease recognition sites and producing linearized vectors having pairs of segments wherein each of said linearized vectors having pairs of segments has a pair of segments of the fragment, one of said pair of segments is located at one end of each of the linearized vectors, and another of said pair of segments is located at another end of each of the linearized vectors.

4. The method of claim **3**, wherein said type IIs recognition sites are recognized by the same type IIs restriction endonuclease.

5. The method of claim **3**, further comprising the step of re-circularizing each of the linearized vectors, by ligating the pair of segments together.

* * * * *

EXHIBIT 8

MARSHALL, GERSTEIN & BORUN LLP

ATTORNEYS-AT-LAW

Kevin M. Flowers, Ph.D.
(312) 474-6615
kflowers@marshallip.com

March 23, 2010

Via U.S. Mail and E-Mail

Nicholas Groombridge, Esq.
Weil, Gotshal & Manges LLP
767 Fifth Avenue
New York, NY 10153
nicholas.groombridge@weil.com

Re: *Life Tech Corp. v. Illumina, Inc.*, Case No. 09-706-RK

Nick:

According to the Court's February 2, 2010 Order, the patentees in this case are to advise the accused infringers of the asserted claims of each patent-in-suit no later than today.

Illumina asserts claims 1-3, 5, and 6 of U.S. Patent No. 6,654,505, claims 1-4 of U.S. Patent No. 6,831,994, claims 1-4 and 7 of U.S. Patent No. 7,232,656, and claims 1-5 of U.S. Patent No. 7,598,035 against Life Technologies Corporation and Applied Biosystems, LLC.

Sincerely,

Kevin M. Flowers

cc: *Via e-mail only*
Jack B. Blumenfeld, Esq. (jblumenfeld@mnat.com)
Maryellen Noreika, Esq. (mnoreika@mnat.com)
Peter Sandel, Esq. (peter.sandel@weil.com)
Jenny C. Wu, Esq. (jenny.wu@weil.com)

EXHIBIT 9

Westlaw.

Not Reported in F.Supp.2d, 2007 WL 2892707 (D.Del.)
(Cite as: 2007 WL 2892707 (D.Del.))

H

Only the Westlaw citation is currently available.

United States District Court,
D. Delaware.
ABBOTT DIABETES CARE, INC., Plaintiff,
v.
DEXCOM, INC., Defendants.
C.A. No. 06-514 GMS.

Sept. 30, 2007.

Mary B. Graham, Rodger Dallery Smith, II, Morris, Nichols, Arsht & Tunnell LLP, Wilmington, DE, for Plaintiff.

Josy W. Ingersoll, Karen Elizabeth Keller, Young, Conaway, Stargatt & Taylor, Wilmington, DE, Brian M. Kramer, David C. Doyle, M. Andrew Woodmansee, Pro Hac Vice, for Defendants.

MEMORANDUM

GREGORY M. SLEET, United States Chief District Judge.

I. INTRODUCTION

***1** On August 17, 2006, Abbott Diabetes Care, Inc. ("Abbott") brought this patent infringement action against DexCom, Inc. ("DexCom"). Presently before the court are the following motions: (1) DexCom's motion to strike Abbott's complaint; (2) Dexcom's motion to consolidate this proceeding with C.A. No. 05-590; and (3) Dexcom's motion to stay this proceeding pending reexamination of the patents-in-suit. (D.I.5.) For the reasons that follow, the court will deny DexCom's motion to strike, grant the motion to consolidate this proceeding with C.A. No. 05-590, and stay the consolidated proceeding until the Patent and Trademark Office (the "PTO") has completed the reexamintion of the seven patents-in-suit.

II. BACKGROUND

A. Procedural Background

There are presently two patent infringement cases before this court in which Abbott and DexCom are parties, the 05-590 action and the present action. In the 05-590 action, Abbott alleges that DexCom infringes its U.S. Patent Nos. 6,175,752 (the "'752 patent"), 6,284,478 (the "'478 patent"), 6,329,161 (the "'161 patent"), and 6,565,509 (the "'509 patent") (collectively, the "Group I Patents"). Looking to add three more patents to the 05-590 lawsuit, namely U.S. Patent Nos. 6,990,366 (the "'366 patent"), 5,899,855 (the "'855 patent"), and 6,134,461 (the "'461 patent") (collectively the "Group II Patents"), Abbott filed an amended complaint pursuant to Federal Rule of Civil Procedure 15(a) in the 05-590 action. (05-590, D.I.55.) DexCom subsequently filed a motion to strike the amended complaint, which the court granted on August 16, 2006. (D.I.61, 05-590.) Abbott responded by filing the present action, alleging patent infringement of the Group II Patents (D.I.1, 06-514), and DexCom filed the three motions presently before the court. Following the opening brief, Abbott filed an answering brief (D.I.9), to which DexCom responded with a reply brief (D.I.10).[FN1]

FN1. Additionally, the parties filed letters (D.I.12, 13) that the court will disregard in making its rulings on the pending motions, because they fail to comply with District of Delaware Local Rule 7.1 .2(b), which states: "Except for the citation of subsequent authorities, no additional papers shall be filed absent Court approval."

B. The Technology at Issue in Abbott's Infringement Actions

Between both actions, Abbott alleges that DexCom infringes a total of seven patents: the Group I Patents, which are directed to methods, systems, and

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

devices for continuously monitoring glucose levels in humans, and the Group II Patents, which are directed to a health monitoring system or an electrochemical sensor. The Group I Patents provide an alternative monitoring system for diabetics, who currently monitor their glucose levels by pricking their fingers to draw blood several times a day. (05-590, D.I. 32, at 3; see '752 Patent, Col. 1, ll 21-26; '509 Patent Col. 1, ll. 21-26.) The technology described in the Group I Patents was invented to address the need for a small and comfortable device that could continuously monitor glucose levels for days at a time, while permitting a patient to engage in normal activities. (See '752 Patent, Col. 2, ll. 1-4; '509 Patent, Col 2., ll. 5-8.) Each of the Group I Patents relate to an aspect of the continuous glucose monitor, which involves implanting a glucose sensor in a patient and monitoring signals over the life of the sensor.[FN2] (D.I. 32, at 3.)

> FN2. The '752 and '509 patents relate to glucose monitoring devices and their methods of use, while the '478 and '161 patents relate to subcutaneous glucose sensors.

***2** The Group II Patents are directed to a health monitoring system comprising a video game unit ('855 patent, Claims 1, 42, and 51), an electrochemical sensor ('461 Patent, Claims 1, 28, and 29), and a method for using an electrochemical sensor ('366 Patent, Claim 1). More specifically, the technology in the '855 patent was invented to take advantage of the processing and graphical presentation capabilities of a portable video game device, when used as a controller and display unit for a glucose monitoring device. ('855 Patent, Col. 4, l. 55-Col. 5, l. 13.) The '366 patent is "applicable to an analyte monitoring system using an implantable sensor for the in vivo determination of a concentration of an analyte, such as glucose or lactate, in a fluid. The sensor can be, for example, subcutaneously implanted in a patient for the continuous or periodic monitoring of an analyte in a patient's interstitial fluid." ('366 Patent, Col. 5, ll. 40-50.) The '461 patent relates to an "analyte sensor which can be used for the in vivo and/or in vitro determination of a level of an analyte in a fluid ... such as glucose or lactate.... One embodiment of the invention is an electrochemical sensor [including] a substrate, a recessed channel formed in a surface of the substrate, and a conductive material disposed in the recessed channel." ('461 Patent, Col 2, ll. 15-27.)

III. DISCUSSION

A. DexCom's Motion to Strike the Complaint

DexCom's motion to strike asserts that the complaint filed in the present case is redundant to the amended complaint that was filed and stricken by the court in the 05-590 action, in view of Rule 12(f) of the Federal Rules of Civil Procedure. Rule 12(f) states, in pertinent part, "[u]pon motion made by a party within 20 days after the service of the pleading upon the party ... the court may order stricken from any pleading any insufficient defense or any redundant, immaterial, impertinent, or scandalous matter." Generally, motions to strike are disfavored. *Am. Standard Life & Accident Ins. Co. v. U.R.L., Inc.*, 701 F.Supp. 527, 532 (M.D.Pa.1988). When ruling on such a motion, "the [c]ourt must construe all facts in favor of the nonmoving party ... and deny the motion if the defense is sufficient under the law." *Id.* Furthermore, courts prefer not to grant a motion to strike "unless it appears to a certainty that ... [the movant] would succeed despite any statement of the facts which could be proved in support of the defense." *Greiff v. T.I.C. Enterprises, L.L.C.*, No. Civ. 03-882, 2004 WL 115553, at * 2 (D.Del. Jan. 9, 2004).

In support of its motion to strike the complaint, DexCom provides two separate arguments: (1) the court's order striking the amended complaint in the 05-590 action required Abbott to ask for leave to file a supplemental amendment, and (2) the claims in the present complaint are redundant of the claims made in the complaint filed in the 05-590 case (the "2005 Complaint"). With respect to DexCom's first argument, the court agrees that the proper vehicle

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

to introduce the Group II Patents into the 05-590 action would have been for Abbott to seek leave of the court under Federal Rule of Civil Procedure 15(d). Nevertheless, nothing in the court's August 16, 2006 Memorandum and Order could be construed as preventing Abbott from seeking relief by filing a new complaint.[FN3] Thus, the court rejects DexCom's first argument, as it is without merit.

FN3. Indeed, the court did not strike Abbott's complaint on the merits but, rather, because it failed to comply with the requirements of Rule 15(d).

***3** In making its second argument, DexCom relies on Rule 12(f), but does not cite pertinent authority to support its position that the Rule empowers the court to strike Abbott's complaint in the present action because it failed to properly amend its complaint to allege infringement of the Group II patents in the 05-590 action.[FN4] The Federal Circuit has squarely addressed the particular legal issue involved here-whether a non-merits based dismissal of a patent claim in one action precludes a plaintiff from bringing that same patent claim in a subsequent infringement action-holding that preclusion does not apply. In *Abbey v. Mercedes Benz of North America, Inc.*, 138 Fed. Appx. 304 (Fed.Cir.2005), the plaintiff filed an amended complaint alleging infringement of a certain patent, which the district court did not consider and dismissed as moot. 138 Fed. Appx. at 306. Subsequently, the plaintiff filed a new action alleging infringement of the same patent. *Id.* The trial court dismissed the new action on res judicata grounds and the plaintiff appealed. The Federal Circuit reversed, holding that claim preclusion did not apply to the patent reasserted in the subsequent action, because "[e]ach patent asserted raises an independent and distinct cause of action," and the trial court did not reach the merits of that patent in the first lawsuit. *Id.* at 307 (quoting *Kearns v. General Motors co.*, 94 F.3d 1553, 1555-56 (Fed.Cir.1996)). Given the foregoing, the court concludes that Abbott's current complaint is not duplicative of complaint in the 05-590 action. Accordingly, the court will deny DexCom's motion to strike Abbott's complaint.

FN4. DexCom cites to three cases to support the general rule that two causes of action are duplicative of each other where both are a consequence of one tortious action, *Munie v. Stag Brewery*, 131 F .R.D. 559, 560 (N.D.Ill.1989), *Davidson v. John Deere & Co.*, 644 F.Supp. 707, 712-13 (N.D.Ind.1986), and *Rogers v. Mount Union Borough*, 816 F.Supp. 308, 318 (M.D.Pa.1993). These cases, however, are inapposite to the present case, because they are not patent cases subject to the Federal Circuit's holdings on the issue, which, as will be discussed, are markedly different.

B. DexCom's Motion to Consolidate this Action with C.A. No. 05-590

DexCom next argues that the court should consolidate this action with the 05-590 action. Pursuant to Federal Rule of Civil Procedure 42(a), courts have the authority to consolidate actions involving a common question of law or fact. *Oracle Corp. v. EpicRealm Licencing, L.P.*, No. Civ. 06-414, 2007 WL 901543, *5 (D.Del. Mar. 26, 2007). Decisions to consolidate cases are discretionary, but often courts balance considerations of efficiency, expenses, and fairness. *United States v. Dentsply Int'l, Inc.*, 190 F.R.D. 140, 142-43 (D.Del.1999). The court "[has] broad power ... to consolidate causes for trial as may facilitate the administration of justice." *EllermanLines, Ltd. v. Atl. & Gulf Stevedores, Inc.*, 339 F.2d 673, 675 (3d Cir.1964).

To support its motion, DexCom argues that "both of Abbott's complaints accuse the same device (DexCom's STS device) of infringing patents which involve closely related subject matter." (D.I. 6, at 14.) DexCom further contends that "the witnesses, documents, and exhibits related to [its] accused device likely will be the same for all seven patents asserted in the two cases." (*Id.*) Finally, DexCom

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

relies on Abbott's admission in its opposition to DexCom's motion to strike the amended complaint in the 05-590 action that it "could have filed a new complaint in this jurisdiction at any time, and that the new case would likely have been consolidated with the current case before this court." (05-590 case, D.I. 67, at 6.)

***4** Abbott advances two separate arguments against DexCom's motion to consolidate: the patented technologies in the Group II Patents are very different from the technologies in the Group I Patents, and consolidating this case with the 05-590 action will delay the advancement of the 2005 action. The court is not persuaded and concludes that the cases should be consolidated. First, with respect to the technologies at issue in each case, the court finds that the claimed subject matter of the Group I Patents is very similar to the subject matter of the Group II Patents.[FN5] Additionally, in each patent action before the court, Abbott alleges that the same DexCom device infringes its patents-the STS device. Moreover, Abbott's admission that a "new case would likely [be] consolidated with the current [05-590] case before this court," (05-590 case, D.I. 67, at 6), supports the court's conclusion that consolidation is warranted. The related technologies at issue in these patents, the fact that all claims of infringement are based on the same device, and that both cases involve the same parties lead the court to conclude that judicial resources likely will be conserved by consolidating these two cases. Thus, the court will grant the motion to consolidate.[FN6]

FN5. In making this finding, the court determined in to which class the PTO has classified each of the respective Group I and Group II Patents. The PTO classified half of the Group I Patents and all of the Group II into class 600. Moreover, Subclass 345 of Class 600 was used to classify two patents in Group I and two patents in Group II. Based on this evidence, it is clear that the PTO found the technologies in the Group I and Group II Patents related. The PTO's classification of most of the patents into the same Class persuades the court that the Group I and II Patents claim related technologies.

FN6. Abbott argues that consolidation will prejudice it, because the Group I Patents are well into the reexamination process, and "the reexamination process will *likely* be finished much sooner than the reexamination [of the Group II Patents]...." (D.I. 9, at 17) (emphasis added). The court disagrees. Abbott's argument assumes that the Group I Patents will emerge from reexamination first. The court finds that this assumption, however, is not enough to demonstrate prejudice.

C. DexCom's Motion to Stay

Finally, DexCom asks this court to stay this proceeding in light of the reexamination by the PTO. Having determined that consolidation of the two actions is proper, the court must now determine whether to stay the entire proceeding until the PTO completes its reexamination of the Group I Patents, or whether to stay the entire proceeding until the PTO completes its reexamination of all seven patents-in-suit. The decision to stay a case is firmly within the discretion of the court. *See Cost Bros., Inc. v. Travelers Indem. Co.*, 760 F.2d 58, 60 (3d Cir.1985). This authority applies equally to patent cases in which a reexamination by the PTO has been requested. *Ethicon, Inc. v. Quigg*, 849 F.2d 1422, 1426-27 (Fed.Cir.1988) (noting that "[c]ourts have inherent power to manage their dockets and stay proceedings, including the authority to order a stay pending conclusion of a PTO reexamination.") (internal citations omitted). In determining whether a stay is appropriate, the court's discretion is guided by the following factors: "(i) whether a stay would unduly prejudice or present a clear tactical disadvantage to the non-moving party; (ii) whether a stay will simplify the issues in question and trial of the case; and (iii) whether discovery is complete and

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

whether a trial date has been set." *Xerox Corp. v. 3 Com Corp.*, 69 F.Supp.2d 404, 406 (W.D.N.Y.1999) (citing cases); *cf. United Sweetener USA, Inc. v. Nutrasweet Co.*, 766 F.Supp. 212, 217 (D.Del.1991) (stating a similar test).

The thrust of DexCom's argument to support its position that staying this case will not prejudice or present a clear tactical disadvantage to Abbott is, "the stay will conserve the resources of the parties and promote the efficient resolution of this case." (D .I. 6, at 21.) Against this reasoning, Abbott argues that DexCom's purpose for ordering the reexamination of the Group II Patents is to delay the process of this case. To support its argument, Abbott quotes *Remington Arms Co., Inc. v. Modern Muzzleloading, Inc.*, No. 2:97CV00660, 1998 WL 1037920 (M.D.N.C. Dec. 17, 1998), for the proposition that: "Generally, courts are reluctant to stay proceedings where a party is using the reexamination process merely as a dilatory tactic." 1998 WL 1037920, at * 3. Abbott then states, "Here, DexCom is doing just that." (D.I. 9, at 14). The court disagrees and finds the facts and posture of *Remington Arms* easily distinguishable from the present case.

***5** In *Remington Arms*, there was record evidence suggesting to the court that the defendant was aware of uncited prior art during fact discovery, but failed to request a reexamination of the patents-in-suit until well after case dispositive motions by both partied were filed and briefed. 1998 WL 1037920, at * 3. Thus, the court found the defendant's unjustified delay in filing the reexamination the most compelling factor in denying the motion to stay. *Id.* Analyzing the facts, the court held, "[u]nder the circumstances, granting the motion to stay would not be judicially efficient, [because] [d]iscovery has closed, a trial date has been set, and both parties have submitted dispositive motions which are presently pending before the court." *Id.* at * 2. The court ruled that "staying the proceedings would only result in a waste of time and judicial resources, especially at this late stage of the litigation." *Id.* In contrast, in the present case, the court finds no such delay, where the court has not yet conducted a Rule 16(2)(b) scheduling conference, no scheduling Order is in place, no discovery has taken place, and little time has yet to be invested in the litigation. Accordingly, staying these proceedings will save time and judicial resources.

Additionally, staying the present case until the reexamination of the all of the patents-in-suit is complete will facilitate and simplify issues for trial. "One purpose of the reexamination procedure is to eliminate trial of that issue (when the claim is canceled) or to facilitate trial of that issue by providing the district court with the expert view of the PTO (when a claim survives the reexamination proceeding)." *Gould v. Control Laser Corp.* 705 F.2d 1340, 1342 (Fed.Cir.1983). Even the *Remington Arms* court noted that it "would surely benefit from the expert opinion of the PTO, and ... awaiting the outcome of the reexamination process could possibly eliminate the need for trial if the claims are canceled...." 1998 WL 1037920, at *3.

Simply put, given that this case is in its early stages and considering the ability of the PTO to narrow and simplify the issues of this case via the reexamination procedure, the court is convinced that a stay is appropriate. Further, as the accompanying order also consolidates this case with the 05-590 action, the court will stay the consolidated case until all of the Group I and Group II Patents complete reexamination.[FN7]

FN7. The court appreciates Abbott's frustration with the delay associated with completing the reexamination process at the PTO. Nevertheless, Abbott's arguments against staying a case pending reexamination are better directed towards Congress than to this court. Despite more than six pages of argument in its brief (D.I. 9, at 10-17), Abbott fails to articulate any specific undue prejudice or clear tactical disadvantage it would suffer. Nor does Abbott provide any meaningful arguments as to

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

why a stay in this case will not simplify the issues in question.

IV. CONCLUSION

For the aforementioned reasons, the court will deny Dexcom's motion to strike the complaint, and grant DexCom's motion to consolidate this case with the 05-590 action and DexCom's motion to stay the proceeding.

ORDER

For the reasons stated in the court's Memorandum of this same date, IT IS HEREBY ORDERED that:

1. The defendant's Motion to Strike (D.I.5) is DENIED.

2. The defendant's Motion to Consolidate (D.I.5) this proceeding with the 05-590 action is GRANTED.

***6** 3. The defendant's Motion to Stay (D.I.5) this proceeding pending the reexamination of the patents in suit is GRANTED.

4. The parties shall notify the court when the PTO issues its reexamination decision on the Group I and Group II Patents.

D.Del.,2007.
Abbott Diabetes Care, Inc. v. Dexcom, Inc.
Not Reported in F.Supp.2d, 2007 WL 2892707 (D.Del.)

END OF DOCUMENT

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

EXHIBIT 10

Westlaw.

Not Reported in F.Supp.2d, 2003 WL 21640372 (D.Del.)
(Cite as: 2003 WL 21640372 (D.Del.))

Only the Westlaw citation is currently available.

United States District Court,
D. Delaware.
ALLOC, INC., a Delaware corporation, et al.,
Plaintiffs,
v.
UNILIN DECOR N.V., a Belgian company, et al.,
Defendants.
No. Civ.A. 03-253-GMS.

July 11, 2003.

MEMORANDUM AND ORDER

SLEET, J.

I. INTRODUCTION

***1** On March 5, 2003, Alloc, Inc. (“Alloc”), Berry Finance N.V. (“Berry”), and Valinge Aluminum AB, (“Valinge”) (collectively “the plaintiffs”) filed a complaint against Unilin Decor, N.V. (“Unilin”) and Quick-Step Flooring, Inc. (“Quick-Step”) (collectively “the defendants”) alleging infringement of U.S. Patent No. 6,516,579 (“the '579 patent”). The '579 patent is the latest in a series of continuation patents that include U.S. Patent Nos. 5,706,621 (“the '621 patent”), 5,860,267 (“the '267 patent”), 6,023,907 (“the '907 patent”), and 6,182,410 (“the '410 patent”).

The '621 patent is currently undergoing reexamination in the United States Patent and Trademark Office (“PTO”). Additionally, the Federal Circuit is considering infringement issues with regard to the '267, '907, and '410 patents after the International Trade Commission (“ITC”) rendered a non-infringement decision in favor of Unilin and against the plaintiffs.

Presently before the court is the defendant's motion to stay litigation of the '579 patent pending the completion of both the '621 reexamination proceedings and the Federal Circuit's decision on the '267, '907, and '410 patents. After consideration of each of the factors involved, and for the reasons detailed below, the court will grant the motion to stay.

II. BACKGROUND

The parties involved in the present action have attempted to resolve their patent infringement issues in many different forums, both in the United States and in Europe. Specifically, in July 2000, Pergo Inc. (“Pergo”), Unilin's licensee, brought a declaratory action in the District of Columbia with regard to the '267, '907, and '621 patents in response to the plaintiffs' threats of infringement litigation. Pergo additionally filed a request for reexamination of the '621 patent in the PTO. This reexamination is currently ongoing. The plaintiffs subsequently filed a complaint in the Eastern District of Wisconsin asserting that Pergo and Unilin infringed the '267 and '907 patents. In response, Unilin filed its own declaratory judgment action in the District of Columbia, alleging that its product did not infringe the '267, '907, and '621 patents.

In December 2000, the plaintiffs initiated a proceeding in the ITC asserting that Unilin infringed the '267, '907, and '410 patents. Upon the filing of the ITC action, all of the district court actions between the two parties concerning the alleged infringement of the '267, '907, and '410 patents were stayed pursuant to 28 U.S.C. § 1659. In November 2001, an ITC Administrative Law Judge (“ALJ”) issued a decision finding that Unilin did not infringe the '267, '907, or '410 patents. The ITC affirmed the ALJ's decision in April 2002. The plaintiffs then appealed to the Federal Circuit, which heard oral argument on that case in March 2003. No decision has yet issued.

In the present case, the '579 patent is the only pat-

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

ent in dispute. However, as the court noted above, it is the latest of the continuation patents that stem from the original '621 patent. The '579 patent has never been reviewed by the PTO, the ITC, or any other court.

III. DISCUSSION

***2** The decision to stay a case is firmly within the discretion of the court. *See Cost Bros., Inc. v. Travelers Indem. Co.*, 760 F.2d 58, 60 (3d Cir.1985). This authority applies equally to patent cases in which a reexamination by the PTO has been requested. *Ethicon, Inc. v. Quigg*, 849 F.2d 1422, 1426-27 (Fed.Cir.1988) (noting that "[c]ourts have inherent power to manage their dockets and stay proceedings, including the authority to order a stay pending conclusion of a PTO reexamination.") (internal citations omitted); *see also Emhart Indus. v. Sankyo Seiki Mfg.*, 3 U.S.P.Q .2d 1889, 1890 (N.D.Ill.1987) (recognizing that, "in passing legislation establishing the reexamination proceeding, Congress stated its approval of district courts liberally granting stays within their discretion."); *Gould v. Control Laser Corp.*, 705 F .2d 1340, 1342 (Fed.Cir.1983) (citing legislative history of reexamination statute).

In determining whether a stay is appropriate, courts are directed to consider the following factors: "(1) whether a stay would unduly prejudice or present a clear tactical disadvantage to the non-moving party; (2) whether a stay will simplify the issues in question and trial of the case; and (3) whether discovery is complete and whether a trial date has been set." *Xerox Corp. v. 3 Comm Corp.*, 69 F.Supp.2d 404, 406 (W.D.N.Y.1999) (citing cases); *cf. United Sweetner USA, Inc. v. Nutrasweet Co.*, 766 F.Supp. 212, 217 (D.Del.1991) (stating a similar test).

In opposition to the defendants' motion to stay, the plaintiffs first argue that, since the '579 patent itself is not at issue in the reexamination proceedings, or in the Federal Circuit appeal, there is no need to stay the case before this court. *See* D.I. 21 at 7. The court must disagree because the plaintiffs cannot credibly argue that the patents are not alike in subject matter, as well as in many of their claims. This is so because, in general, "a continuing application is one filed during the pendency of another application which contains at least part of the disclosure of the other application and names at least one inventor in common with that application." *Transco Products, Inc. v. Performance Contracting, Inc.*, 38 F.3d 551, 555 (Fed.Cir.1994). Thus, a continuation application "claims the same invention claimed in an earlier application, although there may be some variation in the scope of the subject matter claimed." *Id.* Indeed, the plaintiffs themselves admit that the patents in question do have some terms in common. *See* D.I. 21 at 10. Therefore, even though the '579 patent does not contain precisely the same claims of the other patents that are under review or reexamination, there is a sufficient correlation among all of the patents for the court to conclude that a stay is appropriate.

Additionally, with regard to the issue of efficiency, it is beyond dispute that the court would benefit from a narrowing of the numerous complex issues relating to claims, which, if clearly defined, would streamline discovery and subsequent litigation. To this end, the reexamination of the '621 patent will greatly serve the purpose of defining the issues in this case. For example, the court will gain the benefit of the PTO's particular expertise in evaluating the prior art. *See Pegasus Development Corp. v. DirecTV, Inc.*, 2003 WL 21105073, *2 (D.Del. May 14, 2003) (citations omitted). Likewise, the court will also benefit from the reexamination process in that (1) many discovery issues relating to prior art may be alleviated; (2) the record of the reexamination likely would be entered at trial; (3) the issues, defenses, and evidence will be more easily limited in pre-trial conferences following a reexamination; and (4) the outcome of the reexamination process may encourage a settlement without further involvement of the court. *Id.* (citations omitted). Such a refinement of the issues will benefit both parties by reducing litigation costs. *See id.* This approach will also best conserve the court's scarce resources.

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

See id. Similar benefits will likewise flow from the Federal Circuit's analysis of the '267, '907, and '410 patents.

***3** The plaintiffs alternatively contend that the motion is premature because the two proceedings that have a potential impact on this case may be decided well before this case reaches the claim interpretation stage. *See* D.I. 21 at 5. However, if the decisions of the PTO and Federal Circuit are imminent, as the plaintiffs suggest, a stay at this time would not unduly burden their case as the stay would then be of short duration.

Finally, the court notes that discovery in this case has not yet begun, nor has a discovery schedule been entered at this time. Likewise, the court has not yet set a trial date. Therefore, the stay will be entered before any party incurs substantial litigation-related expenses.

IV. CONCLUSION

In light of the above considerations, the court concludes that a stay at this point in the case would not unduly prejudice the plaintiffs or create for them a clear tactical disadvantage. Indeed, a stay will allow the issues before the court to be further simplified and defined to the benefit of the parties, as well as the court.

For the foregoing reasons, IT IS HEREBY ORDERED that:

1. The Defendants' Motion to Stay Pending the Reexamination by the U.S. Patent and Trademark Office and Ruling by the United States Court of Appeals for the Federal Circuit (D.I.15) is GRANTED.

2. The parties shall advise the court of any decision that results from the PTO's reexamination of the '621 patent and any decision that results from the Federal Circuit's consideration of the '267, '907, and '410 patents within thirty (30) days of the date of each decision.

3. The Plaintiffs' Motion to Strike Portions of the Answer and Complaint (D.I.11) is DISMISSED, without prejudice, and with leave to re-file should it become necessary following the stay.

D.Del.,2003.
Alloc, Inc. v. Unilin Decor N.V.
Not Reported in F.Supp.2d, 2003 WL 21640372 (D.Del.)

END OF DOCUMENT

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

EXHIBIT 11

Westlaw.

Not Reported in F.Supp.2d, 2008 WL 3889539 (E.D.Pa.)
(Cite as: 2008 WL 3889539 (E.D.Pa.))

H

Only the Westlaw citation is currently available.

United States District Court,
E.D. Pennsylvania.
CROSS ATLANTIC CAPITAL PARTNERS, INC.
v.
FACEBOOK, INC. and Thefacebook, LLC.
Civil Action No. 07-2768.

Aug. 18, 2008.

Frederick A. Tecce, McShea, Tecce, PC, Patrick J. Keenan, Thomas J. Duffy, Duffy & Partners, Philadelphia, PA, for Cross Atlantic Capital Partners, Inc.

Craig W. Clark, Heidi L. Keefe, Mark R. Weinstein , Sam C. O'Rourke, White & Case, LLP, Palo Alto, CA, Dennis P. Mccooe, Joel L. Dion, Alfred W. Zaher, Blank, Rome, Comisky & McCauley, LLP, Philadelphia, PA, for Facebook, Inc. and Thefacebook, LLC.

ORDER-MEMORANDUM

JOHN R. PADOVA, District Judge.

***1 AND NOW,** this day of August, 2008, upon consideration of Defendants' Motion to Stay Proceedings (Docket Entry 179) and all responses thereto, **IT IS HEREBY ORDERED** that the Motion is **GRANTED** with leave to the parties to seek to lift the stay after the United States Patent and Trademark Office has issued its decision on whether to grant reexamination, or for other good and sufficient reasons that result from a change in circumstances.

On July 21, 2008, Defendants filed a request for inter partes reexamination of United States Patent No. 6,519,629 with the United States Patent and Trademark Office ("PTO") pursuant to 37 U.S.C. § 311. Concomitant therewith, Defendants move to stay this litigation pending the result of that request.

A district court is not required to stay a proceeding pending reexamination. *Viskase Corp. v. Am. Nat'l Can Co.*, 261 F.3d 1316, 1328 (Fed.Cir.2001). However, "[o]ne purpose of the reexamination procedure is to eliminate trial of that issue (when the claim is canceled) or to facilitate trial of that issue by providing the district court with the expert view of the PTO (when a claim survives the reexamination proceeding)." *Gould v. Control Laser Corp.*, 705 F.2d 1340, 1342 (Fed.Cir.1983). Thus, granting a stay is favored. *See Alltech, Inc. v. Cenzone Tech, Inc.*, Civ. A. No. 06-153, 2007 WL 935516 (S.D.Cal. March 21, 2007) (holding that "[t]here is a liberal policy in favor of granting motions to stay proceedings pending the outcome of reexamination proceedings") (quoting *ASCII Corp. v. STD Entm't USA, Inc.*, 844 F.Supp. 1378, 1381 (N.D.Cal.1994)). Stays are particularly appropriate when the reexamination result might assist the court in making a validity determination or would eliminate the need to make an infringement determination. *Id.* (citing *In re Cygnus Telecomm. Tech., LLC Patent Litig.*, 385 F.Supp.2d 1022, 1023 (N.D.Cal.2005).

District courts have listed the following factors to determine whether a stay is appropriate: (1) whether a stay would unduly prejudice or present a clear tactical disadvantage to the nonmoving party; (2) whether a stay will simplify the issues in question and trial of the case; and (3) whether discovery is complete and whether a trial date has been set. *In re Laughlin Prods. Inc.*, 265 F.Supp.2d 525, 530-31 (E.D.Pa.2003); *Sabert Corp. v. Waddington North Am., Inc.*, Civ. No. 06-5423, 2007 WL 2705157, *6 (D.N.J. Sept. 14, 2007) (citing *Alltech*, 2007 WL 935516 at *2 (quoting *Cygnus*, 385 F.Supp.2d at 1023); *Xerox Corp. v. 3Com Corp.*, 69 F.Supp.2d 404, 406 (W.D.N.Y.1999); *ASCII Corp.*, 844 F.Supp. at 1380 (citing *GPAC, Inc. v. D.W.W. Enter., Inc.*, 144 F.R.D. 60, 66 (D.N.J.1992)). We find, applying these factors, that granting a stay

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

pending the PTO's decision to grant reexamination is appropriate.[FN1]

FN1. Plaintiff's preliminary argument, that under 37 U.S.C. § 318 the stay motion is premature, is not substantial. Section 318 states that "[o]nce an order for inter partes reexamination has been issued," a patent owner may obtain a stay of pending litigation which involves the patent undergoing reexamination. The Section speaks only to the timing that the patent owner follows to request a stay, not the third party requester in the inter partes reexamination proceeding. Further, as Facebook notes, courts faced with the issue regularly decide stay motions upon the filing of the request for reexamination, staying the civil action at least until the PTO makes its reexamination decision. *See PBI Performance Prods., Inc. v. NorFab Corp.*, Civ. 05-4836, 2007 WL 81065, * 1 (E.D.Pa. Jan.5, 2007) (Bartle, J.) (noting that action was placed on suspense docket and ordered stayed pending a decision by the PTO whether to reexamine plaintiff's patent)); *Alltech, Inc. v. Cenzone Tech, Inc.*, Civ. 06-153, 2007 WL 935516, *1 n. 2 (S.D.Cal. Mar.21, 2007) (assuming for purposes of ruling on stay motion that the PTO would grant the request because the PTO grants approximately 90% of reexamination requests (citing Paul Morgan & Bruce Stoner, *Reexamination v. Litigation-Making Intelligent Decisions in Challenging Patent Validity*, 86 J. Pat. & Trademark Office Soc'y 441, 459 (2004))); *Pass & Seymore, Inc. v. Hubbell, Inc.*, Civ. A. No. 07-272, 2007 WL 2172648, at *12 (N.D.N.Y. July 23, 2007) (holding that stay was warranted while the PTO made its initial determination; if reexamination was denied, plaintiff could move to lift stay); *Ralph Gonnocci Revocable Living Trust v. Three M Tool & Mach., Inc.*, Civ. A. No. 02-74796, 2003 WL 22870902, at *4 (E.D.Mich. Oct.7, 2003) (same); *but see DUSA Pharms., Inc. v. River's Edge Pharms, LLC*, Civ. A. No. 06-1843, 2006 WL 1320049 (D.N.J. May 15, 2006) (denying the defendant's request for a stay citing § 318 without distinguishing that § 318 speaks only the to patent owner's right to request a stay after the reexamination decision has been issued, and does not by its terms limit the third party requester).

A. Prejudice to Plaintiff

Defendants argue that Plaintiff will suffer no prejudice from a stay because it is undisputed that it: 1) has never sold or marketed any products that embody the '629 Patent; 2) does not compete with Defendants and thus will not be harmed by alleged infringement during the reexamination process; and 3) its damage claim is based on a reasonable royalty which will not be affected by the stay because damages run from the date the Complaint was filed. Plaintiff, while not directly addressing these assertions, argues that having expended substantial resources over the last year preparing for trial, it would be unduly prejudiced by a stay. It also argues that the lapse of time during reexamination could result in loss of evidence and the fading of witness memory.

***2** We find that Defendants have demonstrated a superior argument on the issue of prejudice. It is undisputed that Plaintiff does not practice the patent in suit, does not compete with Defendants, and the delay caused by reexamination will not diminish its measure of damages. On the other hand, Plaintiff's prejudice argument is weak. Having to expend resources to prepare for trial is the natural consequence of its having filed the lawsuit. Fact witness testimony has already been memorialized in depositions and expert witness opinions have been exchanged. The intervening period should not overly burden either parties' ability to try the case.[FN2]

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

FN2. Plaintiff also argues that Defendants filed their reexamination request with the PTO to gain a tactical advantage. The test the courts have articulated for determining prejudice, however, is not whether the moving party will gain a tactical advantage but whether the non-movant will suffer a clear tactical disadvantage. We find that Plaintiff has not shown that it will suffer a tactical disadvantage.

B. Simplification of the Issues for Trial.

This factor favors granting the stay. There are only three possible outcomes from the reexamination process and all three will simplify the issues remaining for trial: 1) the patent is found invalid and cancelled-resulting in no issue remaining for trial; 2) the patent is amended limiting the scope of its claims-and limiting the scope of claims that Defendants could possibly have infringed; and 3) the patent is reaffirmed-removing from the trial Defendants' invalidity defense. Defendants, as the parties that requested the reexamination, are bound by the results of the reexamination. *See* 37 U.S.C. § 315(c).

Plaintiff argues that the reexamination procedure will not resolve issues of priority or Defendants' affirmative defense of inequitable conduct. While this is true, it does not speak to the purpose of the reexamination procedure-patent validity-a threshold issue in this lawsuit. If the invention was not patentable, Defendants' other affirmative defenses are immaterial.

C. Timing of the Motion.

This factor favors denying the stay in the sense that discovery has been completed, and summary judgment motions have been filed of record. Our current scheduling order provides for pre-trial memoranda to be filed in September, a final pre-trial conference on September 19, and a trial date of October 20, 2008. Clearly, granting a stay will disrupt this schedule. However, the expeditious movement of this case toward trial does not mean that granting the stay will unreasonably delay final adjudication of the case.

The reexamination process will take several months. Nonetheless, Defendants argue that this factor favors granting the stay. They note that the case has only been pending for only one year, trial remains three months away, and pre-trial preparations have not yet begun. Because a stay will conserve judicial resources and save the parties substantial resources for trial preparation because many trial issues may be mooted, Defendants conclude that the timing of the motion is not a ground for denying it. Plaintiff argues that the case is in its late stage and argues that other courts have denied requests for stays on this basis. *See e.g.*, *Soverain Software LLC v. Amazon.com, Inc.*, 356 F.Supp.2d 660, 663 (E.D.Texas 2005); *Alltech, Inc.*, 2007 WL 935516, at *4.

***3** Although this action is proceeding toward its conclusion in this court, the factors, on balance, favor granting the stay at least until the PTO makes its initial determination whether to conduct the reexamination. Plaintiff has not made a good argument on the prejudice prong and the reexamination will clearly simplify the issues remaining in the case. Staying further litigation until the PTO makes its decision whether to grant reexamination will permit the PTO to exercise its statutorily granted authority to make the initial Office action on the merits of the reexamination. At that point we can revisit whether the stay should be continued to the end of the reexamination process. If reexamination is denied, there may be no good reason not to proceed; if reexamination is granted, we would have the benefit of the PTO's initial reasoning on the patentability issues to inform our decision on the merits of continuing the stay to the conclusion of the reexamination proceedings.

For these reasons, the stay is **GRANTED** with leave to the parties to seek lifting of the stay after the PTO has issued its decision on whether to grant

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

reexamination, or for other good and sufficient reasons that result from a change in circumstances.

E.D.Pa.,2008.
Cross Atlantic Capital Partners, Inc. v. Facebook, Inc.
Not Reported in F.Supp.2d, 2008 WL 3889539 (E.D.Pa.)

END OF DOCUMENT

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

EXHIBIT 12

Westlaw.

Not Reported in F.Supp.2d, 2001 WL 125340 (D.Del.)
(Cite as: 2001 WL 125340 (D.Del.))

C

Only the Westlaw citation is currently available.

United States District Court, D. Delaware.
GIOELLO ENTERPRISES LTD., Plaintiff,
v.
MATTEL, INC., Defendant.
No. C.A. 99-375 GMS.

Jan. 29, 2001.

Philip A. Royner, Potter, Anderson & Corroon LLP, Wilmington, DE, James G. Goggin, Verrill & Dana LLP, Portland, Maine, for Plaintiff, of counsel.

Robert W. Whetzel, Chad M. Shandler, Richards, Layton & Finger, Wilmington, DE, Peter E. Heuser, David A. Fanning, Charles H. DeVoe, Kolisch, Hartwell, Dickinson, McCormick, & Heuser, Portland, Oregon, for Defendants, of counsel.

ORDER

SLEET, J.

***1** On June 11, 1999, the plaintiff, Gioello Enterprises Ltd. (“Gioello”), filed a complaint against Mattel, Inc. (“Mattel”) claiming infringement of U.S. Patent 4,546,434 (the “ '434 patent”). Mattel answered and filed a counterclaim which Gioello, in turn, answered. Both parties filed motions which are currently pending. Mattel filed a request with the U.S. Patent and Trademark Office (the “PTO”) on November 9, 2000 for an reexamination of claims 1-3 of the '434 patent. The PTO granted Mattel's request on December 21, 2000, and issued a schedule for statements from the parties (D.I.104). Currently before the court is Mattel's motion to stay proceedings pending reexamination (D.I.84).[FN1] Upon consideration of the parties' submissions, the court will grant Mattel's motion and stay the proceedings until further notice.

FN1. Although Mattel filed its motion before the PTO granted its request, the court will consider the PTO's actions.

In deciding whether to stay the proceedings, the court's discretion is guided by the following factors: (1) whether a stay would unduly prejudice Gioello or present a clear tactical advantage for Mattel, (2) whether a stay will simplify the issues, and (3) whether discovery is complete and whether a trial date has been set. *See Xerox Corp v. 3Comm Corp.*, 69 F.Supp.2d 404, 406 (W.D.N.Y.1999) (citing cases); *cf. United Sweetner USA, Inc. v. Nutrasweet Co.*, 766 F.Supp. 212, 217 (D.Del.1991) (stating a similar test).

The PTO's recent decision to reexamine the '434 patent will simplify the issues in this case and focus the litigation. Numerous courts have cited a number of advantages of granting a stay pending PTO reexamination: (1) all prior art presented to the court at trial will have been first considered by the PTO with its particular expertise, (2) many discovery problems relating to the prior art can be alleviated, (3) if patent is declared invalid, the suit will likely be dismissed, (4) the outcome of the reexamination may encourage a settlement without further involvement of the court, (5) the record of the reexamination would probably be entered at trial, reducing the complexity and the length of the litigation, (6) issues, defenses, and evidence will be more easily limited in pre-trial conferences and (7) the cost will likely be reduced both for the parties and the court. *See, e. g., Braintree Laboratories, Inc.*, Civ. A. No. 96-2459-JWL, 1997 WL 94237, at *9 (D.Kan. Feb. 26, 1997); *Hamilton Indus. v. Midwest Folding Products Mfg.*, Civ. A. No. 89-C-8689, 1990 WL 37642, at *1-*2 (N.D.Ill. March 20, 1990) (citing cases). All of these potential advantages are present, to some degree, if the court imposes a stay of the proceedings pending the outcome of the PTO's reexamination.

Not staying the proceedings runs the risk of incon-

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

sistent adjudications or issuance of advisory opinions. Presently, this case is scheduled for trial on April 13, 2001. According to the PTO's order granting the request for reexamination, it is possible that submission of statements will not be complete until April 12, 2001.[FN2] Additionally, the outstanding motions for summary judgment by Mattel claim invalidity and non-infringement-two issues the PTO's decision could render moot. Since the court must decide the summary judgment motions well in advance of trial, it would have to address the arguments raised before the PTO. Such a situation raises resource questions. As one court noted:

> FN2. The PTO's order of December 21, 2000 states that Gioello has up to two months to submit an optional response. If Gioello submits a response, Mattel has up to two months to submit a reply. Since no extensions will be granted, the court believes April 12, 2001-16 weeks from the date of the order is the maximum time allowable for written submissions to the PTO.

> ***2** ... if the parties continue to litigate the validity of the claims in this Court [sic], and the PTO subsequently finds that some or all of the claims in issue here are invalid, the Court [sic] will have wasted time and the parties will have spend additional funds addressing an invalid claim or claims. Thus, although the denial of a stay can have no effect whatsoever on past events, the grant of a stay will maximize the likelihood that neither the Court [sic] nor the parties expend their assets addressing invalid claims.
> *Softview Computer Products Corp. and Ergo View Technologies Corp. v. Haworth, Inc.*, No. 97 Civ. 8815 KMW HBP, 2000 WL 1134471, at *3 (S.D.N.Y. Aug. 10, 2000). Since the PTO cannot stay the reexamination once a request has been granted, the court's issuance of a stay is the only way to avoid the potential for conflict. *See Hamilton*, 1990 WL 37642, at *2 (citing *Ethicon, Inc. v. Quigg*, 849 F.2d 1422 (Fed.Cir.1988).

The court finds the prejudice to Gioello is slight, if any. Gioello claims it is prejudiced both by Mattel's dilatory tactics in not requesting an examination earlier and by it spending money on discovery that is nearly complete. Leaving aside timing arguments, the court finds no merit in this position. First, Gioello is not selling or actively licensing goods or services related to the '434 patent; money damages is an adequate remedy for any delay in redress. Second, both Mattel and Gioello have spent money on discovery. Third, Mattel is entitled by law to request a reexamination. *See* 35 U.S.C. §§ 302-307; 37 C.F.R. § 1.525. The fact that the request was granted means the PTO deems the '434 patent worthy of reexamination. It is not for the court to second guess the PTO's decision to reassess the prior art.

Given the possibility that the PTO's reexamination could materially affect the issues in this case, the court will deny Mattel's motions for summary judgment and Gioello's motion to strike without prejudice.[FN3] Although the could will hold a status conference in late April or early May, 2001, the parties should advise the court of any earlier developments in the PTO's reexamination.

> FN3. Upon the entry of a new scheduling order, the parties are free to re-file their motions.

Therefore, IT IS HEREBY ORDERED that:

1. Mattel's motion to stay the proceeding pending reexamination (D.I.84) is GRANTED. The proceedings are stayed from the date of this order until further notice.

2. Mattel's motion for summary judgment on invalidity (D.I.87) is DENIED without prejudice.

3. Mattel's motion for summary judgement on noninfringement (D.I.89) is DENIED without prejudice.

4. Gioello's motion to strike (D.I.91) is DENIED without prejudice.

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

5. The parties shall advise the court of any decision that results from the PTO's reexamination of the '434 patent.

D.Del.,2001.
Gioello Enterprises Ltd. v. Mattel, Inc.
Not Reported in F.Supp.2d, 2001 WL 125340 (D.Del.)

END OF DOCUMENT

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

EXHIBIT 13

Westlaw.

Slip Copy, 2010 WL 181375 (E.D.Pa.)
(Cite as: 2010 WL 181375 (E.D.Pa.))

C

Only the Westlaw citation is currently available.

This decision was reviewed by West editorial staff and not assigned editorial enhancements.

United States District Court,
E.D. Pennsylvania.
HERAEUS ELECTRO-NITE CO., LLC, Plaintiff
v.
VESUVIUS USA CORP., Defendant.
Civil Action No. 09-2417.

Jan. 11, 2010.

Jason A. Snyderman, Lewis Wayne Schlossberg, Blank Rome LLP, John D. Simmons, Panitch Schwarze Belisario & Nadel LLP, Philadelphia, PA, for Plaintiff.

Alan R. Silverstein, Eric J. Evain, Rudolf E. Hutz, Zhun Lu, Steven A. Nash, Connolly Bove Lodge & Hutz LLP, Wilmington, DE, Donald M. Satina, Vesuvius USA Corp., Pittsburgh, PA, for Defendant.

EXPLANATION

ANITA B. BRODY, District Judge.

***1** Plaintiff Heraeus Electro-Nite Co., LLC ("HEN") owns two patents for instruments used to measure the temperature of molten metal. On May 27, 2009, HEN filed this action claiming that Defendant Vesuvius USA Corp. ("Vesuvius") makes, uses, offers, or sells products that infringe its patents. On October 1, 2009, Vesuvius filed a request with the United States Patent and Trademark Office (the "PTO"), asking it to reexamine the two HEN patents at issue in this matter. Immediately thereafter, Vesuvius filed a motion with this Court to stay the instant proceeding pending the PTO's reexamination. After a conference on October 15, 2009, I denied Vesuvius's motion for a stay pending reexamination.

On December 10, 2009, Vesuvius filed a renewed motion to stay the proceedings pending reexamination. Vesuvius claimed two key developments since I last addressed the issue: (1) Vesiuvius stated that it will not make or sell the accused product for the remaining life of the patents in suit, and (2) the PTO granted the reexamination requests for both patents.

District courts "have broad discretion to manage their dockets, including the power to grant a stay of proceedings." *Procter & Gamble Co. v. Kraft Foods Global, Inc.*, 549 F.3d 842, 849 (Fed.Cir.2008). In determining whether to stay an action pending the reexamination of a patent by the PTO, courts consider "(1) whether a stay will unduly prejudice or present clear tactical disadvantage to the nonmoving party, (2) whether a stay will simplify the issues in question and the trial of the case, and (3) whether discovery is complete and whether a trial date has been set." *Spa Syspatronic, AG v. VeriFone, Inc.*, No. 07-416, 2008 U.S. Dist. LEXIS 34223, at *5,2008 WL 1886020 (E.D.Tex. Apr. 24, 2008).

Because Vesuvius has withdrawn the allegedly infringing product from the market, there is little chance of prejudice to HEN. To be sure, courts have been reluctant to grant stays where, as here, the parties are direct competitors. *See NIDEC Corp. v. LG Innotek Co., Ltd.*, No. 07-108, 2009 U.S. Dist. LEXIS 46123, at *4, 2009 WL 3673433 (E.D.Tex. Apr. 3, 2009). In such situations, stays are denied where there is concern that the patent owner will be irreparably harmed because the accused product will continue to gain market share during the pendency of the stay. *See, e.g., Innovative Office Prods, v. SpaceCo, Inc.*, No. 05-4037, 2008 U.S. Dist. Lexis 67500, at *11, 2008 WL 4083012 (E.D.Pa. Aug. 28, 2008). Because Vesuvius has withdrawn the accused product, there is no

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

risk of irreparable harm accruing during the stay. HEN can be adequately compensated with money damages. Thus, any "delay caused by reexamination will not diminish [HEN's] measure of damages." *Cross Atl. Capital Partners, Inc. v. Facebook, Inc.*, No. 07-2768, 2008 WL 3889539, at *2 (E.D.Pa. Aug.18, 2008). The absence of prejudice to HEN weighs in favor of a stay.

The second factor is whether a stay will simplify the issues in question. FN1 There is a significant likelihood that the reexamination process will simplify the issues in question by altering the claims. PTO statistics suggest that, for *ex parte* reexaminations, the PTO cancels claims in 11 percent of cases and modifies them in 64 percent of cases. United States Patent and Trademark Office *Ex Parte* Reexamination Filing Data, June 30, 2009, http://www.uspto.gov/web/patents/documents/ex_parte.pdf. Thus, while it is true that, as HEN notes, 89 percent of reexaminations conclude with at least some of the patent's claims surviving the reexamination process, many of those claims emerge from reexamination in a different form. Moreover, "whether or not the PTO ultimately amends or invalidates a patent's claims during reexamination, the PTO's reexamination provides the Court with an expert funneling of the issues for trial." *Spa Syspatronic*, 2008 U.S. Dist. LEXIS 34223, at *5, 2008 WL 1886020.

FN1. I note that the possibility that the reexamination will be incomplete when the patents' lives expire has no effect on this factor. Reexamination will continue after a patent's expiration, so long as the patent remains inside the "period of enforceability," which is determined by adding six years to the date on which the patent expires. Manual of Patent Examining Procedure § 2211. Thus, there is no risk that if the reexamination is pending when the patents expire, the PTO will simply decline to address the issues, eroding the potential benefits of reexamination.

***2** HEN also argues that *ex parte* reexaminations typically fail to simplify the issues in litigation because the moving party is free to assert the same issues of invalidity that the PTO already addressed. *See NIDEC Corp.*, 2009 U.S. Dist. LEXIS 46123, at * 16, 2009 WL 3673433. Although Vesuvius's ability to relitigate the validity of the patents at trial after reexamination reduces some of the potential for simplification, the benefits of simplification still accrue for any claims that the PTO cancels or amends. Moreover, the delay associated with the stay will be minimized by reexamination procedures that accelerate the reexamination process for patents involved in litigation. *See Spa Syspatronic*, 2008 U.S. Dist. LEXIS 34223, at *10, 2008 WL 1886020.

Finally, the third issue is whether discovery is complete and whether a trial date has been set. This litigation remains at a very early stage in the proceedings; discovery is open until August 31, 2010. This factor weighs in favor of a stay, as any simplification of the issues will also reduce the costs of discovery.

Thus, I will grant Vesuvius's renewed motion for a stay pending reexamination.

E.D.Pa.,2010.
Heraeus Electro-Nite Co., LLC v. Vesuvius USA Corp.
Slip Copy, 2010 WL 181375 (E.D.Pa.)

END OF DOCUMENT

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

EXHIBIT 14

Westlaw.

Not Reported in F.Supp.2d, 2003 WL 21105073 (D.Del.)
(Cite as: 2003 WL 21105073 (D.Del.))

H

Only the Westlaw citation is currently available.

United States District Court,
D. Delaware.
PEGASUS DEVELOPMENT CORPORATION and Personalized Media Communications, L.L.C., Plaintiffs
v.
DIRECTV, INC., Hughes Electronics Corporation, Thompson Consumer Electronics, Inc., and Philips Electronics North America Corporation, Defendants.
No. Civ.A. 00-1020-GMS.

May 14, 2003.

MEMORANDUM AND ORDER

SLEET, J.

***1** AND RELATED COUNTERCLAIMS

I. INTRODUCTION

On December 4, 2000, Pegasus Development Corporation ("Pegasus") and Personalized Media Communications, L.L.C. ("PMC") filed a complaint against several defendants, alleging infringement of six patents, including U.S. Patent Nos. 4,965,825 ("the '825 patent") and 5,335,277 ("the '277 patent"). Since that time, the original scheduling order has been revised several times. Currently, fact discovery is scheduled to close on August 22, 2003, and a trial is scheduled for February of 2004.

On February 4, 2003 and March 14, 2003, respectively, the defendant Thomson Consumer Electronics, Inc. ("Thomson") filed with the Patent and Trademark Office ("PTO") a request for *ex parte* reexaminations of the '825 and '277 patents. The request for reexamination of the '825 patent was granted on April 10, 2003.[FN1] Presently before the court is a joint motion by the defendants to stay the litigation pending the completion of the patent reexaminations (D.I.459). After careful consideration of the parties' submissions, and for the reasons detailed below, the court will grant the motion.

FN1. The court is not yet aware of a decision by the PTO regarding reexamination of the '277 patent.

II. DISCUSSION

The decision to stay a case is firmly within the discretion of the court. *Cost Bros., Inc. v. Travelers Indem. Co.*, 760 F.2d 58, 60 (3d Cir.1985). This authority applies equally to patent cases in which a reexamination by the PTO has been requested. *Ethicon, Inc. v. Quigg*, 849 F.2d 1422, 1426-27 (Fed.Cir.1988) ("Courts have inherent power to manage their dockets and stay proceedings, including the authority to order a stay pending conclusion of a PTO reexamination.") (internal citation omitted); *see also Emhart Indus. v. Sankyo Seiki Mfg.*, 3 U.S.P.Q.2d 1889, 1890 (N.D.Ill.1987) ("[I]n passing the legislation establishing the reexamination proceeding, Congress stated its approval of district courts liberally granting stays within their discretion."); *Gould v. Control Laser Corp.*, 705 F.2d 1340, 1342 (Fed.Cir.1983) (citing legislative history of reexamination statute). In determining whether a stay is appropriate, the court is guided by the following factors: "(1) whether a stay would unduly prejudice or present a clear tactical disadvantage to the non-moving party; (2) whether a stay will simplify the issues in question and trial of the case; and (3) whether discovery is complete and whether a trial date has been set." *Xerox Corp v. 3 Comm Corp.*, 69 F.Supp.2d 404, 406 (W.D.N.Y.1999) (citing cases); *cf. United Sweetner USA, Inc. v. Nutrasweet Co.*, 766 F.Supp. 212, 217 (D.Del.1991) (stating a similar test).

In this case, there are two plaintiffs, four defend-

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

ants, and several counter claimants, as well as six patents comprising dozens of claims. In addition, the written submissions in this case have been particularly voluminous; the briefing on claim construction alone, for example, constitutes 576 pages. *See* Report and Recommendation of Special Master Regarding Claim Construction at 2 (citing "copious briefing"). In these ways, the present suit is quite complex, although, perhaps, not extraordinarily so. The greater context of this suit is extraordinary, however: the plaintiffs have filed more than 300 related patent applications based upon an original patent application filed in 1981 and supplemented in 1987. Together, these applications contain an estimated 10,000 claims. Furthermore, as observed by the Special Master appointed in this case, the 1987 application alone constitutes over 300 columns of patent text and "is, by any measure, an extremely complex document." *Id.* at 2. These related applications may become relevant to the present case in respect to several issues including claim construction, enablement, adequacy of written description, indefiniteness, and inequitable conduct. *See id.* at 21. Thus, in this case, more than many, the court would benefit from a narrowing of the issues.

***2** The reexamination process will serve this purpose. For example, the court will gain the benefit of the PTO's particular expertise, in that all prior art presented to the court will have been first considered by that agency. *See Braintree Laboratories, Inc. v. Nephron-Tech, Inc.*, 1997 WL 94237, at *9 (D.Kan.1997); *Hamilton Indus., Inc. v. Midwest Folding Products Mfg.*, 1990 WL 37642, at *1-2 (N.D.Ill.1990). Other potential efficiencies resulting from the reexamination process are numerous: (1) many discovery problems relating to the prior art may be alleviated; (2) the record of the reexamination likely would be entered at trial, reducing the complexity and length of the litigation; (3) the issues, defenses, and evidence will be more easily limited in pre-trial conferences following a reexamination; (4) the outcome of the reexamination process may encourage a settlement without further involvement of the court; and (5) if the patent is declared invalid, the suit likely will be dismissed as to that patent. *Id.* These efficiencies will result in a reduced cost of litigation for the parties and more effective utilization of the limited resources of the court. *Id.*

Thus, a stay may result in a simplification or reduction of issues for the court's consideration, or it may dispense with the litigation entirely. These are considerable economies indeed, particularly in this case. Given the involved prosecution history of the various patents-in-suit and hundreds of related patents, the number of claim terms at issue, the inordinate amount of prior art references, and the PTO's conclusion that all of the challenged claims warrant reexamination, the court finds particular merit in permitting an additional layer of review by the PTO before expending further judicial resources. *See Digital Magnetic Systems, Inc. v. Ainsley*, 213 U.S.P.Q. 290, 290 (W.D.Okla.1982) ("Congress enacted the reexamination procedure to provide an inexpensive, expedient means of determining patent validity which, if available and practical, should be deferred to by the courts."); *Softview Computer Products Corp. v. Haworth, Inc.*, 2000 WL 1134471, at *3 (S.D.N.Y.2000) ("[T]he grant of a stay will maximize the likelihood that neither the Court nor the parties expend their assets addressing invalid claims."). Furthermore, the court notes that discovery is not complete, and the trial, although scheduled, is some nine months in the future. In light of all these factors, and considering that the reexamination process will proceed "with special dispatch," 35 U.S.C. 305, the court concludes that a stay is the most compelling alternative.

The court recognizes that a stay will cause further delay in a case that has suffered several delays already, as well as considerable distress to the plaintiffs. The court is sensitive to the plaintiffs' right to have their day in court. Nonetheless, for the reasons already mentioned, the court is convinced that a stay is appropriate in this particular case. In addition, the court reminds the plaintiffs that they affirmatively invoked the rights of the patent stat-

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

ute; they can hardly be heard now to complain of the rights afforded others by that same statutory framework. Thomson is legally entitled to invoke the reexamination mechanism, and the PTO has determined that reexamination is warranted. There is nothing facially untoward in that. Moreover, the court notes that if, after reexamination, the plaintiffs' patents are again upheld, the plaintiffs' rights will only be strengthened, as the challenger's burden of proof becomes more difficult to sustain. *See Custom Accessories, Inc. v. Jeffrey-Allan Indus., Inc.*, 807 F.2d 955, 961 (Fed.Cir.1986) (holding that upon reissue, the burden of proving invalidity is 'made heavier') (quoting *Interconnect Planning Corp. v. Feil*, 774 F.2d 1132, 1139 (Fed.Cir.1985)). In this light, and given the particular circumstances of this case, the court cannot find that the prejudice to the plaintiffs is undue.

***3** Objecting to a stay, the plaintiffs also have complained of dilatory conduct by the defendants, who, in turn, have accused the plaintiffs of "burying" the PTO in claims and prior art references. *See, e.g.*, Mem. of Plaintiffs in Opp. to Defs.' Joint Motion to Stay (D.I.488) at 5-22 (detailing ways in which the defendants allegedly "have repeatedly acted to complicate and delay the resolution of this litigation"); Defs.' Joint Brief in Support of Motion to Stay (D.I.460) at 4 ("Many of the Harvey patents had vast numbers of cited prior art references of record, effectively burying the most relevant ones.") and 7 ("[I]t appears that PMC sought to 'overwhelm' the PTO and the Courts.").

As a brief response to the accusation of dilatory conduct, the court notes that Thomson's request for reexamination of the '825 patent comprised 2,610 pages; its request regarding the '277 patent totaled 4,736 pages. It is presumed that such an effort requires an enormous expenditure of time and other resources; thus, the timing of Thomson's reexamination requests does not, necessarily, reflect undue delay. Furthermore, as noted above, Thomson was legally entitled to invoke the reexamination procedure when it did. As to the defendants' repeated complaint that the plaintiffs overwhelmed the PTO with prior art references during prosecution of the patents-in-suit, the implications of such alleged conduct will be explored at another time in the litigation, if necessary. At this stage in the process, the court is satisfied that the PTO has found "substantial new questions of patentability" raised by each of the cited references, and has determined that all of the challenged claims of the '825 patent necessitate a reexamination. *See* PTO's Decision Granting Reexamination, Supp. Appendix to Defs.' Joint Brief in Support of Motion to Stay (D.I.467) at 138. Although the court regrets a further delay in the present case, it is confident that the advantages of a stay outweigh the costs.

III. CONCLUSION

Because the PTO's reexamination of one or more of the patents-in-suit may materially affect the issues in this case, the court will grant the defendants' motion to stay. The case is stayed pending a disposition of the PTO's reexamination of patent '825, and will be stayed pending reexamination of the '277 patent, if applicable. All pending motions will be denied without prejudice; the parties may refile them following the stay and upon the entry of a new scheduling order, if applicable.

Thus, for the aforementioned reasons, IT IS HEREBY ORDERED that:

1. The defendants' Motion to Stay Pending Reexamination by the U .S. Patent and Trademark Office (D.I.459) is GRANTED. The proceedings are stayed from the date of this order until further notice.

2. The parties shall advise the court of any decision that results from the PTO's reexamination of the '825 patent, and any other decision of the PTO regarding reexamination of any of the other patents-in-suit.

***4** 3. The plaintiffs' Motion for Leave to Assert Claim 15 of U.S. Patent 4,965,825 (D.I.399) is

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

DENIED without prejudice.

4. Thomson's Motion to Dismiss or in the Alternative for Summary Judgment (D.I.376) is DENIED without prejudice.

5. Phillips' Motion to Dismiss for Lack of Standing (D.I.396) is DENIED without prejudice.

D.Del.,2003.
Pegasus Development Corp. v. Directv, Inc.
Not Reported in F.Supp.2d, 2003 WL 21105073 (D.Del.)

END OF DOCUMENT

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

EXHIBIT 15

Westlaw.

Not Reported in F.Supp.2d, 2009 WL 528564 (D.Del.)
(Cite as: 2009 WL 528564 (D.Del.))

C
Only the Westlaw citation is currently available.

United States District Court,
D. Delaware.
WALL CORPORATION, Plaintiff,
v.
BONDDESK GROUP, L.L.C., and Bonddesk Trading, L.L.C., Defendant.
C.A. No. 07-844 GMS.

Feb. 24, 2009.

Karen Elizabeth Keller, Young, Conaway, Stargatt & Taylor, Wilmington, DE, Alfonso Chan, Michael W. Shore, Patrick A. Traister, Pro Hac Vice, for Plaintiff.

Mary B. Graham, Morris, Nichols, Arsht & Tunnell, Wilmington, DE, Michael A. Jacobs, Rita F. Lin, Pro Hac Vice, for Defendant.

ORDER

CHIEF, District Judge.

***1** 1. On December 26, 2007, Wall Corporation ("Wall") brought this patent infringement action against BondDesk Group, LLC and BondDesk Trading, LLC (collectively, "BondDesk"). Presently before the court is BondDesk's motion to stay this proceeding pending reexamination of the patent-in-suit. (D.I.11). For the reasons set forth below, the court will grant the motion.

2. The complaint alleges that BondDesk infringes United States Patent No. 7,231,363 (the "'363 patent"), entitled Method and System for Rebrokering Orders in a Trading System.

3. On June 3, 2008, BondDesk filed a request for *inter partes reexamination* of the '363 patent. On that same date, BondDesk filed the motion to stay that is presently before the court. The parties completed briefing on BondDesk's motion on June 30, 2008.

4. The Patent and Trademark Office (the "PTO") granted BondDesk's reexamination petition on August 22, 2008. In addition, the PTO issued an office action, on August 22, 2008, rejecting all fifteen claims of the '363 patent. (See D.I. 15 Ex. B.)

5. On November 10, 2008, the court held a scheduling conference with the parties. On November 20, 2008, the court entered a scheduling order (D.I.23), setting the close of fact discovery for September 28, 2009, and a trial date of July 12, 2010.

6. The decision to stay a case is firmly within the discretion of the court. *See Cost Bros., Inc. v. Travelers Indem. Co.*, 760 F.2d 58, 60 (3d Cir.1985). This authority applies equally to patent cases in which a reexamination by the PTO has been requested. *Ethicon, Inc. v. Quigg*, 849 F.2d 1422, 1426-27 (Fed.Cir.1988) (noting that "[c]ourts have inherent power to manage their dockets and stay proceedings, including the authority to order a stay pending conclusion of a PTO reexamination.") (internal citations omitted). In determining whether a stay is appropriate, the court's discretion is guided by the following factors: "(i) whether a stay would unduly prejudice or present a clear tactical disadvantage to the non-moving party; (ii) whether a stay will simplify the issues in question and trial of the case; and (iii) whether discovery is complete and whether a trial date has been set." *Xerox Corp. v. 3 Com Corp.*, 69 F.Supp.2d 404, 406 (W.D.N.Y.1999) (citing cases); *cf. United Sweetener USA, Inc. v. Nutrasweet Co.*, 766 F.Supp. 212, 217 (D.Del.1991) (stating a similar test).

7. The first factor the court considers is prejudice to Wall and tactical advantage to BondDesk. As previously noted, the PTO has issued a first office action rejecting all claims of the '363 patent. The reexamination of the ' 363 patent is ongoing, however, and the court cannot predict the outcome or timing of

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

the proceedings. Given the delay in the PTO proceedings, the court finds that the first factor weighs slightly against granting a stay.

8. The court next considers whether a stay will simplify the issues in question and trial of the case. If the court accedes to BondDesk's request and grants the motion to stay, BondDesk will be estopped from making the same invalidity arguments that it makes to the PTO during the reexamination proceeding (or any invalidity arguments that it could have made to the PTO) in this case, because the reexamination is *inter partes.* 35 U.S.C. § 315(c). The '363 patent is the only patent asserted by Wall in this lawsuit. Therefore, this estoppel will resolve many of the invalidity issues and streamline the litigation. As such, this factor weighs in favor of staying the case.

***2** 9. Finally, the court considers whether discovery is complete and whether a trial date has been set. As previously mentioned, the court has entered a scheduling order, which sets a deadline for the parties to complete discovery and sets a trial date. BondDesk filed the present motion, however, early in the case, prior to the court's scheduling conference and entry of a scheduling order. Accordingly, this factor weighs in favor of granting a stay.

10. Weighing all of the considerations as it must, the court concludes that the interests in staying the litigation outweigh any interests in proceeding at this time. As discussed, the only factor that weighs against staying the litigation is the delay that might enure in the reexamination process.[FN1] The court finds that this delay does not, by itself, amount to undue prejudice. *See Research in Motion, Ltd. v. Visto Corp.*, 545 F.Supp.2d 1011, 1012 (N.D.Cal.2008) (granting a stay pending reexamination where the delay caused by reexamination was the only prejudice identified). Therefore, IT IS HEREBY ORDERED that:

FN1. Wall argues that it will be prejudiced and BondDesk will gain a tactical advantage if the court grants the stay because (1) documents will be lost, witnesses' memories will fade, and witnesses will become unavailable; (2) a delay of the trial will force Wall to wait to collect infringement damages and there are no guarantees that BondDesk will be solvent in the future; and (3) the '363 patent inventors will be prevented from enforcing the patent against other infringers. The court has considered these arguments and rejects them as speculative or irrelevant to its determination of prejudice and tactical advantage.

1. The defendant's Motion to Stay the Proceeding Pending *Inter Partes Reexamination* (D.I.11) is GRANTED.

2. The parties shall notify the court when the PTO issues its reexamination decision on the '363 patent.

D.Del.,2009.
Wall Corp. v. BondDesk Group, L.L.C.
Not Reported in F.Supp.2d, 2009 WL 528564 (D.Del.)

END OF DOCUMENT

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

EXHIBIT 16

Westlaw

Not Reported in F.Supp.2d, 2001 WL 34368283 (D.Del.)
(Cite as: 2001 WL 34368283 (D.Del.))

C

Only the Westlaw citation is currently available.

United States District Court,
D. Delaware.
COGNEX CORPORATION, Plaintiff,
v.
NATIONAL INSTRUMENTS CORPORATION, Defendant.
No. Civ.A. 00-442-JJF.

June 29, 2001.

Neal C. Belgam, and Dale R. Dube, of Blank Rome Comisky & McCauley LLP, Wilmington, Delaware, George M. Sirilla, G. Paul Edgell, and William P. Atkins, of Pillsbury Winthrop LLP, Washington, D.C., for Plaintiff, of counsel.

William J. Marsden, Jr., and John T. Meli, Jr., of Fish & Richardson P.C., Wilmington, Delaware, Frank E. Scherkenbach, David M. Barkan, and John V. Picone III, of Fish & Richardson P.C., Menlo Park, California, for Defendant, of counsel.

MEMORANDUM OPINION

FARNAN, J.

***1** Pending before the Court in this patent infringement action is Cognex Corporation's Motion To Stay Or, In The Alternative, For An Extension Of The Deadlines In This Case (D.I.84). By its Motion, Plaintiff, Cognex Corporation (“Cognex”), requests a stay or, in the alternative, a three month delay in the proceedings in this case, because it has requested a reexamination of the patent in suit by the United States Patent and Trademark Office (“PTO”). By letter dated May 29, 2001, Cognex advised the Court that the PTO granted Cognex's request for reexamination of United States Patent No. 5,481,712 (the “ '712 Patent”). Cognex contends that reexamination of the '712 Patent will narrow and/or resolve the issues in this case. Specifically, Cognex contends that if the '712 Patent is declared unpatentable, it is likely that the patent litigation will be dismissed. On the other hand, if the PTO finds the '712 Patent patentable over the prior art, Cognex contends that the PTO decision might encourage the parties to settle this action.

Defendant, National Instruments Corporation (“National Instruments”), opposes any stay in this case. Specifically, National Instruments contends that a stay would cause National Instruments severe prejudice because: (1) the litigation schedule and trial date would be delayed causing National Instruments additional expense; (2) Cognex would be able to continue to hold the specter of litigation over National Instruments and its customers; and (3) Cognex has already forced National Instruments to spend over a million dollars defending itself in this action before requesting the stay, despite the fact that Cognex had the materials necessary to seek reexamination earlier.

After reviewing the parties' positions with respect to the instant motion and the applicable law, the Court concludes that a stay and/or extension is not warranted in this case. Accordingly, for the reasons discussed, the Court will deny Cognex's Motion To Stay Or, In The Alternative, For An Extension Of The Deadlines In This Case.

DISCUSSION

The decision to grant or deny a stay is within the court's broad range of discretionary powers. *Dentsply International, Inc. v. Kerr Manufacturing Co.*, 734 F.Supp. 656, 658 (D.Del.1990) (citations omitted). In determining whether a stay is appropriate, the court should “weigh the competing interests of the parties and attempt to maintain an even balance.” *Id.* In weighing the interests involved, courts are generally guided by such factors as (1) whether a stay would unduly prejudice or present a clear

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

tactical advantage to the non-movant; (2) whether a stay will simplify the issues raised by the parties; and (3) whether discovery is complete and a trial date has been set. *Gioello Enterprises Ltd. v. Mattel, Inc.*, 2001 WL 125340 (D.Del. Jan.29, 2001); *United Sweetener USA, Inc. v. Nutrasweet Co.*, 766 F.Supp. 212, 217 (D.Del.1991). In balancing these factors, courts must be particularly mindful of the consequences of the stay on other parties. *Dentsply International*, 734 F.Supp. at 658 (recognizing that Court must consider whether "there is 'even a fair possibility' that the stay would work damage on another party") (citations omitted). Where a stay will forestall the trial date agreed upon by the parties, this Court has required the party requesting the stay to "make a showing of 'a clear case of hardship or inequity' before the Court can enter a stay order." *Id.* (citing *Gold v. Johns-Manville Sales Corp.*, 723 F.2d 1068, 1076 (3d Cir.1983)).

***2** In this case, discovery was scheduled to close on May 4, 2001, less than three weeks before Cognex filed the instant Motion To Stay, and trial is scheduled for October 23, 2001.[FN1] Accordingly, Cognex must demonstrate a clear case of hardship or inequity before the Court will enter an order staying this action.

> FN1. Although discovery has since been extended until July 2001 by stipulation between the parties (D.I.106), the trial date in this case has not been changed.

After reviewing the parties' arguments, the Court concludes that Cognex cannot demonstrate a clear case of hardship or inequity, and that National Instruments would be prejudiced if the Court were to grant a stay in this case. Cognex primarily contends that reexamination will simplify the issues in this case such that the litigation may be settled or dismissed and the parties' expenses significantly reduced. However, as National Instruments points out, Cognex's complaint alleges a variety of claims which are not linked to the patent infringement claim, including claims of copyright and trademark infringement and unfair competition, all of which will require a trial.

In addition, Cognex contends that the reexamination may result in changed claims, which would cause this action to be litigated twice, wasting the Court's and the parties' resources. However, the Court is unpersuaded by Cognex's contention. The trial in this case is scheduled for October 2001, and the PTO granted Cognex's request for reexamination in May 2001. Although Cognex disputes the figures provided by National Instruments concerning the pendency of applications in the PTO because National Instruments relies on original applications rather than reexamination applications, even Cognex's figures suggest that the median reexamination time is 16 months. Thus, given the current time tables for action in the PTO, the Court believes that the trial in this case will likely be completed prior to any action by the PTO. (D.I. 87 at Exh. G; D.I. 94, Exh. D).

Further, any hardship incurred by Cognex as a result of its request for reexamination is, in part, a result of Cognex's own making. National Instruments contends and Cognex has not disputed, that Cognex has had at least some of the documents it presented to the PTO in its request for reexamination for quite some time. National Instruments identified specific prior art references in its Answer to the Complaint in June 2000, yet Cognex waited until six months before the scheduled trial date in this case to seek reexamination of the '712 Patent.[FN2] Accordingly, the Court cannot conclude that Cognex has demonstrated a clear case of hardship or inequity justifying a stay in this case. *Dentsply*, 734 F.Supp. at 659 ("The Court will not elevate [a party's] failure to address its concerns in a timely fashion to an example of hardship warranting a stay."); *see also Remington Arms Company, Inc. v. Modern Muzzleloading, Inc.*, 1998 WL 1037920 (D.N.C. Dec.17, 1998) (denying stay where trial date set and defendant's delay in requesting reexamination with PTO was unjustified given that defendant knew of the prior art forming its request for reexamination well prior to its reexamination request).

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

FN2. Although Cognex contends that National Instruments did not produce many of these documents until much later, National Instruments also contends and Cognex has not disputed, that some of the documents Cognex presented to the PTO were in Cognex's possession as early as 1990, well before the inception of this litigation. Thus, it appears to the Court that Cognex still could have filed its request for reexamination at an earlier date.

***3** Moreover, given the late stage of Cognex's request, the Court finds that any delay in the trial date scheduled for this case would severely prejudice National Instruments. The parties have conducted extensive discovery in Delaware, California, Texas, Washington, D.C. and Massachusetts based on a schedule coinciding with the October 23 trial date. National Instruments has scheduled its experts and made trial support accommodations in Delaware based upon the October 23 trial date. Any deviation from the October trial date at this late stage in the litigation would necessarily prejudice National Instruments. *See Wayne Automation Corp. v. R.A. Pearson Co.,* 782 F.Supp. 516 (E.D.Wash.1991) (denying plaintiff's motion for stay where parties conducted extensive discovery and trial date was set). Accordingly the Court will deny Cognex's request for a stay.

As for Cognex's request for alternative relief in the form of a three month extension, the Court notes that the discovery deadlines in this case have been extended by stipulation of the parties. This scheduling extension should address Cognex's time concerns without necessitating a change in the October 2001 trial date. Accordingly, at this time, the Court will deny Cognex's request for a three month extension.

CONCLUSION

For the reasons discussed, Cognex Corporation's Motion To Stay Or, In The Alternative, For An Extension Of The Deadlines In This Case will be denied.

An appropriate Order will be entered.

D.Del.,2001.
Cognex Corp. v. National Instruments Corp.
Not Reported in F.Supp.2d, 2001 WL 34368283 (D.Del.)

END OF DOCUMENT

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

EXHIBIT 17

Westlaw.

Not Reported in F.Supp.2d, 2007 WL 551615 (D.Minn.)
(Cite as: 2007 WL 551615 (D.Minn.))

H

Only the Westlaw citation is currently available.

United States District Court,
D. Minnesota.
CARD TECHNOLOGY CORPORATION,
Plaintiff,
v.
DATACARD CORPORATION, Defendant,
and
DataCard Corporation, Counterclaim Plaintiff,
v.
NBS Technologies, Inc. and Card Technology Corporation, Counterclaim Defendants.
Civil No. 05-2546 (MJD / SRN).

Feb. 21, 2007.

Mark R. Privratsky, David A. Allgeyer and Christopher R. Sullivan, Lindquist & Vennum, P.L.L.P, Minneapolis, MN, for Plaintiff and Counterclaim Defendants.

Jeffrey J. Keyes, John B. Lunseth II, and James J. Long, Briggs and Morgan, P.A., Minneapolis, MN, for Defendant and Counterclaim Plaintiff.

ORDER

MICHAEL J. DAVIS, United States District Judge.

***1** The above-entitled matter comes before the Court upon the Report and Recommendation of United States Magistrate Judge Susan Richard Nelson dated February 2, 2007. No objections have been filed to that Report and Recommendation in the time period permitted.

Based on the Report and Recommendation of the Magistrate Judge, and all of the files, records and proceedings herein,

IT IS HEREBY ORDERED that:

1. Plaintiff's motion to stay certain of Defendant's counterclaims pending the PTO's reexamination proceedings (Doc. No. 80) is GRANTED IN PART (to the extent that it seeks to stay the Patent Counterclaims (Counts I & II) and DENIED IN PART (to the extent that it seeks to stay the Nigerian Counterclaim (Count V).

REPORT AND RECOMMENDATION

SUSAN RICHARD NELSON, United States Magistrate Judge.

This matter comes before the undersigned United States Magistrate Judge on Plaintiff's Motion to Stay (Doc. No. 80). The matter has been referred to the undersigned for a Report and Recommendation pursuant to 28 U.S.C. § 636(b) and District of Minnesota Local Rule 72.1.

Plaintiff moves to stay Defendant's patent infringement and business tort counterclaims pending reexamination of the patents underlying the infringement counterclaims. For the reasons stated below, the Court recommends granting the motion in part and denying it part.

I. FACTUAL AND PROCEDURAL HISTORY

Plaintiff filed this action in November 2005 alleging infringement of three of its patents ("the Patent Claims"). (Doc. No. 1.) Defendant's Answer, filed in January 2006, included the following counterclaims: (1) claims for infringement of three of its patents (Counts I, II & III), (2) a claim seeking a declaratory judgment that Plaintiff's patents were unenforceable and not valid (Count IV, "the declaratory judgment claim"), and (3) a claim for tortious interference with a prospective business advantage in Nigeria regarding the sale of products and services involving technology claimed in the various patents (Count V, the "Nigerian Counterclaim"). (Doc. No. 11.) FN1 The technology at issue with

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

respect to the parties' patents generally involves so-called "smart cards," that is, cards such as credit cards that include additional personalized information.

> FN1. Although Defendant filed its Counterclaims against NBS Technologies, Inc. as well as against Plaintiff Card Technology Corporation, for ease of reference this Court will refer to the Counterclaim Defendants collectively as "Plaintiff."

Due to a partial settlement, only two of Defendant's patents-U.S. Patent No. 5,266,781 ("the '781 Patent") and U.S. Patent No. 6,474,925 ("the '925 Patent") (collectively, "the Counterclaim Patents")-remain at issue.[FN2] Defendant generally alleges that Plaintiff infringes the '781 Patent and the '925 Patent ("the Patent Counterclaims"). (Doc. No. 11.)

> FN2. Based upon stipulated settlement agreements, the claims and defenses regarding Defendant's third patent (Count III) were dismissed with prejudice. (Doc. Nos. 71 & 73.)

Under the Second Amended Pretrial Scheduling Order of September 7, 2006, the parties are still in the early stage of pre-trial proceedings. (Doc. No. 64 (ordering joint claim construction statement by March 13, 2007, with fact discovery to close on September 14, 2007, and expert discovery to close on October 12, 2007).) Trial is scheduled for April 2008.(*Id.*)

***2** On August 31, 2006, Plaintiff filed applications with the United States Patent and Trademark Office ("the PTO") for a reexamination of the entirety of the '925 Patent and of claims 1-3, 20-22 and 25 of the '781 Patent. In October 2006, the PTO granted those requests. Accordingly, on November 28, 2006, Plaintiff moved for a stay of the Patent Counterclaims (Counts I & II) and the Nigerian Counterclaim (Count V). (Doc. No. 80.)

II. DISCUSSION

A. Granting A Stay Is Within This Court's Discretion

Federal courts have the "inherent power to manage their dockets and stay proceedings, including the authority to order a stay pending conclusion of a PTO reexamination." *Ethicon, Inc. v. Quigg*, 849 F.2d 1422, 1426-27 (Fed.Cir.1988) (internal citations omitted). A court is not required to stay such actions, the decision to do so being within the court's sound discretion. *NTP, Inc. v. Research in Motion, Ltd.*, 397 F.Supp.2d 785, 787 (E.D.Va.2005).

Yet courts "routinely" grant such stays where the circumstances warrant. *CNS, Inc. v. Silver Eagle Labs, Inc.*, 2004 WL 3631121, at *1 (D.Minn. Nov. 29, 2004) (granting stay where case was "in the early stages of discovery and there are obvious efficiencies to be gained" by awaiting PTO's decision). *Accord ASCII Corp. v. STD Entertainment USA, Inc.*, 844 F.Supp. 1378, 1381 (N.D.Cal.1994) (noting "that there is a liberal policy in favor of granting [such] motions to stay"); *GPAC, Inc. v. D.W.W. Enterprs., Inc.*, 144 F.R.D. 60, 62 (D.N.J.1992) (stating that "Congress noted its approval of district courts liberally granting stays" pending reexamination). Indeed, Congress intended that the PTO's reexamination proceedings "could settle validity disputes more quickly and less expensively than the often protracted litigation involved in such cases" and "would allow courts to refer patent validity questions to the expertise of the Patent Office." *Patlex Corp. v. Mossinghoff*, 758 F.2d 594, 602 (Fed.Cir.1985).

B. At This Stage Of The Proceedings, A Stay Of The Patent Counterclaims Is Warranted In Light Of The Pending Reexamination

Plaintiff claims that a stay of the Patent Counterclaims pending the reexamination will serve the interests of judicial efficiency and economy "by

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

avoiding unnecessary and duplicative proceedings to consider the validity of the patents, to construe numerous challenged claim terms, and to analyze infringement contentions asserting claims that will not survive the reexamination." (Mem. at 5.) Plaintiff also contends that a stay will not prejudice Defendant. (*Id.* at 7; Reply Mem. at 2, 9-12.)

Defendant counters that a stay of the Patent Counterclaims is not warranted because: (1) the reexamination does not concern all of the claims of one of its Counterclaim Patents, (2) staying the Counterclaims would operate as a bifurcation of Defendant's claims from Plaintiff's Patent Claims, (3) the motion to stay "is a transparent effort to gain a tactical advantage" in light of the minimal remaining life of one of the Counterclaim Patents, (4) there is no guarantee that the reexamination will alter the Counterclaim Patents, and (5) Plaintiff "simply waited too long" to seek reexamination. (Mem. at 1-5.)

***3** Courts generally consider the following factors in deciding whether a stay would be appropriate:

> (1) whether a stay would unduly prejudice or present a clear tactical disadvantage to the non-moving party; (2) whether a stay will simplify the issues in question and trial of the case; and (3) whether discovery is complete and whether a trial date has been set.

Xerox Corp. v. 3Com Corp., 69 F.Supp.2d 404, 407 (W.D.N.Y.1999), *cited in*, *Vdata, LLC v. Aetna, Inc.*, 2006 WL 3392889, at *5 (D.Minn. Nov. 21, 2006), and *3M Innovative Props. Co. v. Dupont Dow Elastomers LLC*, 2005 WL 2216317, at *1 (D.Minn. Sept. 8, 2005). *Accord Agar Corp. v. Multi-Fluid, Inc.*, 983 F.Supp. 1126, 1128 (S.D.Tex.1997) (listing factors of (1) PTO's expertise, (2) stay's effect on litigation, and (3) stage of litigation when stay sought).[FN3]

> FN3. Defendant relies on several decisions from other federal districts in support of its argument that "[a]dditional factors" should be considered. (Mem. at 8-9.) Some of these cases merely address factors, or aspects of factors, already included in the widely-accepted general standard. *E.g. Accent Designs, Inc. v. Jan Jewelry Designs, Inc.*, 1994 WL 121673 (S.D.N.Y.1994) (addressing whether discovery is well under way); *Output Technology Corp. v. Dataproducts Corp.*, 22 U.S.P.Q.2d 1072 (W.D.Wash.1991) (same). Other cases erroneously assume reexamination proceedings can benefit the litigation only if the PTO would cancel all the claims at issue. *Enrotech Corp. v. Autotech Corp.*, 1990 WL 37217 (N.D.Ill.1990) (noting that parties would be back before the court if "the reexamination will not resolve everything"). In any event, this Court adheres to the generally-accepted framework as articulated in *Xerox Corp. v. 3Com Corp.*, 69 F.Supp.2d 404, 407 (W.D.N.Y.1999).

1. The Reexamination Could Reasonably Alter The Parameters Of The Litigation And, In Any Event, Will Almost Surely Facilitate This Court's Resolution Of The Patent Counterclaims

As Plaintiff notes (Mem. at 10-11), courts have identified numerous advantages to staying patent litigation during reexamination by the PTO:

> (1) All prior art presented to the Court will have been first considered by the PTO, with its particular expertise.
>
> (2) Many discovery problems relating to the prior art can be alleviated by the PTO examination.
>
> (3) In those cases resulting in effective invalidity of the patent, the suit will likely be dismissed.
>
> (4) The outcome of the re-examination may encourage a settlement without the further use of the Court.

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

(5) The record of re-examination would likely be entered at trial, thereby reducing the complexity and length of the litigation.

(6) Issues, defenses and evidence will be more easily limited in pre-trial conferences after a re-examination.

(7) The cost will likely be reduced both for the parties and the Court.

GPAC, Inc., 144 F.R.D. at 63, *cited in*, *Arctic Cat, Inc. v. Injection Research Specialists, Inc.*, 2003 WL 22047872, at *2 (D.Minn. Aug. 29, 2003). *Accord Vdata, LLC v. Aetna, Inc.*, 2006 WL 3392889, at *6 (D.Minn. Nov. 21, 2006); *3M Innovative Props. Co. v. Dupont Dow Elastomers LLC*, 2005 WL 2216317, at *2 (D.Minn. Sept. 8, 2005). In short, common sense counsels that it is usually prudent for a court to await the PTO's reassessment of the patents at issue before resuming litigation over the validity, enforceability or infringement of those patents.

Defendant argues that it is far from certain that a reexamination will result in any modification of particular claims, much less the total cancellation of entire patents. (Mem. at 10-13.) Granted, such results are not predictable. But the fact remains that it is generally prudent for a court to await the PTO's decision on such matters unless countervailing factors weigh to the contrary. *See CNS, Inc. v. Silver Eagle Labs, Inc.*, 2004 WL 3631121, at *1 (D.Minn. Nov. 29, 2004) ("Notwithstanding the absence of two claims from the '362 Patent reexamination, the Court concludes that deference to that proceeding is the best use of the judicial and party resources in this case.").

***4** Moreover, even if the reexamined patent emerges unscathed and completely intact, the Court has the benefit of the PTO's expert guidance on the prior art it addressed. *Softview Computer Products Corp. v. Ergo View Technologies Corp.*, 2000 WL 1134471, at *3 (S.D.N.Y. Aug. 10, 2000) ("[Even] if the reexamination proceeding reaffirms all the claims as issued, the Court will then have the benefit of the PTO's expert analysis of the prior art that allegedly invalidates or limits the claims.") (citing Note, "Reexamination Reality: How Courts Should Approach a Motion to Stay Litigation Pending the Outcome of Reexamination," 66 *Geo. Wash. L.Rev.* 172, 180-81 & nn. 82-83 (1997)). In short, a stay is usually warranted unless other factors outweigh the potential benefits of awaiting the outcome of reexamination.

Here, this Court finds that there likely are benefits in waiting for the results of the PTO's reexamination of the Counterclaim Patents. Thus, the issue becomes whether other factors outweigh these benefits.

2. A Stay Of The Patent Counterclaims Will Not Prejudice Defendants Or Otherwise Place Them At A Disadvantage

Defendant raises a variety of related arguments that a stay of its Patent Counterclaims would prejudice it. Defendant first argues that a blanket stay of the two Patent Counterclaims would be unfair because not all of the claims of those patents will be reexamined. (Mem. at 12, 16.) It next argues that a stay of its Counterclaims would permit Plaintiff's Patent Claims to go forward, thereby effectively bifurcating the case. (Mem. at 7-8, 19.) Defendant asserts that the potentially lengthy delay will thus preclude its ability to obtain injunctive relief as well as damages. (Mem. at 13-16.) Finally, Defendant suggests that Plaintiff's motion to stay is simply a "stalling tactic." (Mem. at 11.)

Granted, a stay by definition involves delay. But not all delay is necessarily prejudicial and here any cost of the delay is likely offset by the gains to be achieved by obtaining the PTO's expert guidance on these matters. Moreover, although Defendant raises the specter of a lengthy and open-ended delay, this Court notes that "[a]ll reexamination proceedings under this section, including any appeal to the Board of Patent Appeals and Interferences, will be

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

conducted with special dispatch within the Office." 35 U.S.C. § 305.[FN4]

> FN4. The PTO's own regulations likewise provide that "[a]ll ex parte reexamination proceedings, including any appeals to the Board of Patent Appeals and Interferences, will be conducted with special dispatch." 37 C.F.R. § 1550(a).

Defendant also objects to Plaintiff's request that even though not all of the claims of the two Counterclaim Patents are being reexamined, the stay should nevertheless encompass the entirety of those two patents, not just the particular claims subject to reexamination. (Mem. at 8.) Although such circumstances might not present the optimal scenario for granting a stay, there is no obvious better alternative. *See CNS, Inc. v. Silver Eagle Labs, Inc.*, 2004 WL 3631121, at *1 (D.Minn. Nov. 29, 2004) (noting lack of "any solutions for an efficient and effective division of the case between" the reexamined claims and the rest). *Cf. Pacesetter Inc. v. Cardiac Pacemakers, Inc.*, 2003 WL 23303473, at *1, *3 (D.Minn. Nov. 19, 2003) (rejecting "option of a partial stay of the two patents that are not in reexamination" as "impracticable, if not impossible"). Given that the reasons for staying the action regarding the claims that are subject to reexamination clearly remain valid, there is no discernible way for this Court to proceed efficiently on only those claims that are not being reexamined.

***5** Defendant next urges that Plaintiff is actually seeking a bifurcation of Defendant's Counterclaims from Plaintiff's Patent Claims. (Mem. at 2, 8.) Defendant suggests that it would be unfairly prejudiced by having to defend against Plaintiff's Patent Claims while not being able to pursue its Counterclaims. (*Id.* at 3, 15.) Defendant further notes that the remaining life of the '781 Patent is "only another four plus years," arguing that a delay will diminish Defendant's ability to recover damages due to Plaintiff's alleged financial problems. (*Id.* at 3.)

Plaintiff counters that its Patent Claims should proceed despite the stay of the Counterclaims because "the instant action is really two distinct patent infringement suits based on patents for distinct technology forced together only by [Defendant's] counterclaims. [Defendant's] patents and [Plaintiff's] patents relate to different aspects of card processing." (Mem. at 9.) Plaintiff thus argues that "[d]iscovery for, claim construction of, and even trial about [Plaintiff's] patents will not, therefore, in any way hinge upon [Defendant's] Counterclaim Patents." (*Id.* at 10.)

This Court rejects the suggestion that bifurcation would necessarily be prejudicial to Defendant.[FN5] Any potential prejudice would seem to arise only where Plaintiff's Patent Claims and Defendant's Patent Counterclaims would be intertwined or overlapping such that one party's claims could not be fully and fairly litigated in isolation from the opposing party's claims.

> FN5. The Court notes that Defendant has indicated that it likely will shortly file with the PTO a petition for the reexamination of the Plaintiff's patents, and thus also file a motion with this Court for a stay of the Patent Claims pending the outcome of that reexamination. But resolution of that issue must await a proper motion to stay pending a reexamination, a petition for which has not yet been filed.

This does not appear to be the case here. Any overlap here seems to be confined to the general nature of the basic underlying technology. The advent of smart cards-which involve an ever-increasing amount of personalized information being coded onto the cards-apparently has resulted in a slower rate of processing card stock into finished smart cards. Plaintiff asserts that the inventions covered by its Patent Claims permit a much higher card processing rate to be achieved by card-personalization machines such as that manufactured by Defendant. (Complaint ¶ 8.)

Defendant's Patent Counterclaims appear to be gen-

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

erally directed to similar technology. The '781 Patent pertains to a modular card processing system that "permits the use of modules which can be assembled in an arbitrary sequence to perform the required card processing functions." '781 Patent, col. 2, ll. 39-42. The '925 Patent generally relates to "a linear personalization machine of simpler, less costly construction," which due to its "more compact" design" permits "card processing lines of shorter length." ' 925 Patent, col. 1, ll. 39-45.

Although the parties' patents thus bear a certain similarity in that they both concern the same basic technology-that is, the manufacture of smart cards-that general similarity is essentially inconsequential in the present context of determining the propriety of granting a stay. It would appear that a jury could fairly decide the Patent Claims separately from the Patent Counterclaims and vice versa. In other words, the relationship between the two parties' respective claims is not such as to require their simultaneous resolution by the same jury. Any loss in terms of inefficiency and duplication appears negligible.

***6** Defendant's opposition to what it characterizes as "bifurcation" is also premised on the remedial consequences it anticipates from having to await the PTO's reexamination. It claims prejudice based on the assertion that the reexamination will take longer than the remaining life of the '781 Patent, thereby depriving it of its "right to injunctive relief." (Mem. at 13-14.) This argument rests largely on speculation. First, this Court cannot assume at this pre-trial juncture that Defendant will prevail on its infringement claims. Second, even assuming it does so prevail, a patentee has no absolute right to a permanent injunction. Even a judgment of infringement does not automatically entitle the patentee to a permanent injunction. *See Ebay Inc. v. Mercexchange, L.L.C.*, --- U.S. ----, 126 S.Ct. 1837, 1839 (2006) (reversing ruling that patentee is generally entitled to permanent injunction and instead applying general four-factor test for such relief).

Moreover, such injunctions necessarily presume that the patent has not expired. The courts are under no obligation, however, to manage infringement litigation so as to ensure that a judgment is entered before the patent expires. If a patent has expired, the patentee is not left without a remedy as it has the right to recover past damages and any loss of the right to exclude through an injunction while the patent is still in effect is offset by the presumably greater amount of past damages recoverable for infringement over the remaining life of the patent.

Defendant also asserts that the currently precarious financial condition of Plaintiff and its corporate parent will "virtually guarantee that [Defendant] can obtain no effective relief on the '781 Patent." (Mem. at 14.) Defendant further contends that Plaintiff is in the process of being taken private by its corporate parent and contends that if forced to await the results of reexamination it "will be left to pursue collection of any judgment, probably in Canada, and long after the corporate entities have been stripped of any remaining value by" the entities acquiring Plaintiff. (Mem. at 15-16.) Any such arguments are not only speculative, they address matters that are beyond this Court's proper decision-making purview. This Court simply cannot alter the exercise of its discretion, much less its application of the law to the facts, in order to facilitate one party's efforts to better navigate the speculative vagaries of the business environment.

Finally, Defendant argues that Plaintiff "unduly delayed in Petitioning for Reexamination of the ' 925 patent," alleging that one of Plaintiff's counsel knew of the prior art cited as the basis for the reexamination petition long before filing that petition or the present motion. (Mem. at 20-21.) Plaintiff contends, however, that it properly waited before filing for reexamination until "it was apparent that [Defendant] was not going to act after being approached by [Plaintiff] with the material prior art." (Mem. at 8; *see* Reply Mem. at 9-10 (asserting it provided Defendant with opportunities "to acknowledge that this prior art invalidates the '781 and '925 patents or at the very least completely precludes the

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

reading of those patents on [Plaintiff's] equipment").) On the present record, this Court cannot impute to Plaintiff any dilatory or otherwise improper motives based solely on the mere span of such time intervals.

***7** In sum, Defendant's claims of prejudice fail to outweigh the inherit benefits of awaiting the PTO's reexamination of the bulk of the Counterclaim Patents.

3. A Stay Is Appropriate At This Stage Of The Litigation Where Discovery Is Not Complete and The Scheduled Trial Date Is Not Impending

The usual reason for denying such a stay is that the case has progressed through the bulk of pre-trial proceedings and is scheduled for trial shortly. *E.g. Xerox,* 69 F.Supp.2d 404, 407 (W.D.N.Y.1999) (noting that case had been pending two years, parties had engaged in substantial discovery and dispositive motion practice, and case was set for pre-trial conference two months later). *See also Agar Corp. v. Multi-Fluid, Inc.*, 983 F.Supp. 1126, 1128 (S.D.Tex.1997) (denying stay where "case could be considered to be in its later stages of litigation" and court already had the benefit of a prior PTO reexamination of one of the patents). No such concern is present here, where the parties are still in the early stages of pre-trial matters. *Cf. ASCII Corp. v. STD Entertainment USA, Inc.*, 844 F.Supp. 1378, 1381 (N.D.Cal.1994) (granting stay where "the parties are in the initial stages of the lawsuit and have undertaken little or no discovery" and "the case has not been set for trial").

C. There Is No Basis To Stay The Nigerian Counterclaim

Plaintiff contends that the Nigerian Counterclaim should also be stayed, arguing that "[t]he basis of the tort counterclaim is sufficiently related to the patent infringement counterclaims as to make a stay of all counterclaims, including the tort counterclaim, required." (Mem. at 13.) Plaintiff asserts that the "tort claim is intertwined with the patent infringement claims." (*Id.*) Plaintiff points out that Defendant included the Nigerian Counterclaim in this action because it, like the Patent Counterclaims, "arise[s] from sales of the same equipment allegedly infringing the Counterclaim Patents." (*Id.* at 14.) Plaintiff also contends that the stay should extend to encompass the Nigerian Counterclaim because that claim rests on supplemental jurisdiction that is premised on the Patent Counterclaims. (*Id.*)

Defendant asserts there is no basis to stay its Nigerian Counterclaim, noting that the PTO will not, of course, "be interpreting, or considering in any way, the Nigerian tort issues in the reexamination process." (Mem. at 5.) Defendant also contends that "[d]elay of the Nigerian Counterclaim would prejudice [its] ability to prosecute the claim and it would adversely impact this court's ability to effectively manage the case." (*Id.* at 6.)

This Court agrees that there is no basis presently to stay the Nigerian Counterclaim.[FN6] That counterclaim alleges that Plaintiff wrongfully interfered with Defendant's reasonable expectation of a business advantage in connection with a deal to supply card personalization equipment and related supplies and services to the Nigerian government. (Answer ¶ 39.) Defendant asserts that it had substantially negotiated such a deal, but refused to pay a last-minute $3 million price increase that it believed actually constituted a bribe. (*Id.* ¶ 43.) Defendant further alleges that Plaintiff then agreed to pay the $3 million fee and accordingly was awarded the contract with the Nigerian government. (*Id.* ¶ 44.) Pursuant to that contract, Plaintiff allegedly sold certain products that Defendant claims infringe its Counterclaim Patents. Defendant thus claims that it would have realized its prospective business advantage had not Plaintiff agreed to pay the bribes, such that Plaintiff's action constituted wrongful interference with that advantage. (*Id.* ¶¶ 50-51.)

FN6. The Court notes that the parties have indicated that a recently-filed arbitration proceeding that questions Plaintiff's own-

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

> ership of the patents on which it premises its present Patent Claims might warrant a stay of the entirety of the present action pending the outcome of that arbitration. But resolution of that issue must await a proper motion to stay pending arbitration, a motion which has yet to be filed.

***8** Although the prospective economic advantage that Defendant in the Nigerian Counterclaim alleges it was wrongfully denied concerns sales of equipment that allegedly infringe Defendant's Counterclaim Patents, this claim turns in no way upon the precise scope of the claims of the patents or even upon the continuing existence of those patents. Rather, the Nigerian Counterclaim essentially rests on whether Plaintiff's actions in entering the contract at issue wrongfully interfered with a business opportunity that Defendant should have otherwise obtained-that is, that Plaintiff wrongfully took sales that should have belonged to Defendant. Although the Nigerian Counterclaim is thus based on sales of equipment, the actionable wrong is not based on an allegation that the equipment sales infringed Defendant's patents. Rather, it is based on the allegation that Plaintiff took the sales from Defendant, an allegation that is independent of whether Defendant possessed valid patents claiming an invention practiced in the products it would have sold. In other words, regardless of what happens in the reexamination proceedings, Defendant could pursue the Nigerian Counterclaim because it simply is not dependent on or intertwined with the Patent Counterclaims so as to require staying it pending reexamination of the Counterclaim Patents.[FN7]

> FN7. The authorities on which Plaintiff relies in support of its argument are readily distinguishable. In *Softview Computer Prods. Corp. v. Haworth, Inc.*, the court had *already* stayed the Plaintiff's "non-patent" claim that "defendant is improperly attempting to enforce a patent that it knows is invalid" for separate reasons independent of the bases for staying the defendant's patent claims pending reexamination. 2000 WL 1134471, at *1 n. 1 (S.D.N.Y. Aug. 10, 2000) (and also noting that such claims are properly stayed because they are "dependent on the outcome of patent validity and infringement issues"). In *Clintec Nutrition Co. v. Abbott Labs.*, 1995 WL 228988 (N.D.Ill. Apr. 14, 1995), the court simply ruled that an alleged infringer's counterclaim for inequitable conduct should also be stayed where the patentee's reissue application warranted a stay of its claims for infringement. *Id.* at *6. In *Polaris Industries, Inc. v. Jerrico Int'l, Inc.*, No. 06-CV-2153 (slip op.), this Court stayed a plaintiff's Lanham Act trade dress claims as well as its patent infringement claims pending the PTO's reexamination of the patent "because of the interconnected nature of the two claims." *Id.* at 9.

Nor does the fact that subject matter jurisdiction over this claim rests upon Section 1367 require, or even weigh in favor of, staying this claim. Plaintiff contends that this Court has jurisdiction over the Nigerian Counterclaim only if that claim derives from the same nucleus of operative facts as does the Patent Counterclaims. (Mem. at 15 (quoting 28 U.S.C. § 1367 (extending jurisdiction to state law claims that are "so related to claims in the action within such original jurisdiction that they form part of the same case or controversy under Article III").)

Granted, such a state law claim generally may be brought in federal court only where it meets the requirements for supplemental jurisdiction under Section 1367. Here, the Patent Counterclaims provide the federal question basis for extending supplemental jurisdiction to the Nigerian Counterclaim. But the fact that the Patent Counterclaims would be stayed would not undermine the jurisdictional basis for the Nigerian Counterclaim. The Patent Counterclaims would still exist-even though stayed-and thus would still support supplemental jurisdiction.[FN8]

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

FN8. Two of the three cases on which Plaintiff relies are inapposite as they concern situations where the district court has *dismissed* the federal question claims on which supplemental jurisdiction was premised. *Jones v. Minn. Dept. of Corrections*, 2006 WL 3102984, at *10 (D.Minn. Oct. 31, 2006) (declining to exercise supplemental jurisdiction over state-law claim after dismissing all claims over which court had original jurisdiction); *Minnesota Ind. Ventures, L.L.C. v. City of Roseville*, 2006 WL 763208, at *5 (D.Minn. March 24, 2006) (same). Although Plaintiff argues based on these cases that this Court should now stay the Nigerian Counterclaim because of the possibility that the Patent Counterclaims would later be dismissed (based on the speculative assumption that the PTO would cancel all claims of the Counterclaim Patents), this Court notes that even assuming such an outcome, it still would not be *required* to then dismiss the Nigerian Counterclaim. *See* 28 U.S.C. § 1367(c)(3) (providing that district court possesses discretion, after dismissing "all claims over which it has original jurisdiction," to retain or dismiss remaining state-law claim). The third case is even less relevant because there the court in fact addressed the state-law claim in light of the fact that it had not dismissed *all* of the claims over which it had original jurisdiction. *Young v. Minn. Dept. of Corrections*, 2006 WL 2670030, at *11-12 (D.Minn. Sept. 18, 2006).

III. CONCLUSION

A stay of Defendant's Patent Counterclaims (Counts I & II) is warranted as the PTO's reexamination of the relevant patents could likely alter the claims at issue and otherwise facilitate this Court's resolution of this action. The other relevant factors-potential prejudice to Defendant and the stage of pre-trial proceedings-do not outweigh the benefit of awaiting the PTO's determination here. There is no basis to stay the Nigerian Counterclaim (Count V), however, because it is not dependent on or otherwise intertwined with the Patent Counterclaims.

IV. RECOMMENDATION

***9** Based on the foregoing, and all the files, records and proceedings herein, IT IS HEREBY RECOMMENDED that:

1. Plaintiff's motion to stay certain of Defendant's counterclaims pending the PTO's reexamination proceedings (Doc. No. 80) be GRANTED IN PART (to the extent that it seeks to stay the Patent Counterclaims (Counts I & II) and DENIED IN PART (to the extent that it seeks to stay the Nigerian Counterclaim (Count V).

D. Minn.,2007.
Card Technology Corp v. DataCard Corp.
Not Reported in F.Supp.2d, 2007 WL 551615 (D.Minn.)

END OF DOCUMENT

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

EXHIBIT 18

Westlaw

Not Reported in F.Supp.2d, 2006 WL 2501494 (E.D.Tex.)
(Cite as: 2006 WL 2501494 (E.D.Tex.))

Only the Westlaw citation is currently available.

United States District Court,
E.D. Texas, Texarkana Division.
ECHOSTAR TECHNOLOGIES CORP., Plaintiff,
v.
TIVO, INC., et. al., Defendant.
No. 5:05 CV 81 DF.

July 14, 2006.

Charles S. Barquist, Martin M. Noonen, Morrison & Foerster LLP, Los Angeles, CA, Damon Michael Young, John Michael Pickett, Young Pickett & Lee, Texarkana, TX, Harold J. McElhinny, Rachel Krevans, Morrison & Foerster LLP, San Francisco, CA, Karl J. Kramer, Marc J. Pernick, Timur S. Engin, Morrison & Foerster, Palo Alto, CA, for Plaintiff.

Christine W. S. Byrd, Crawford Maclain Wells, Adam S. Hoffman, Jeffrey L. Arrington, Morgan Chu, Perry M. Goldberg, Richard M. Birnholz, Irell & Manella, Los Angeles, CA, Samuel Franklin Baxter, Attorney at Law, Marshall, TX, Babak Redjaian, Irell & Manella, Newport Beach, Newport Beach, CA, Garret Wesley Chambers, Kristi Jean Thomas, McKool Smith, Dallas, TX, for Defendant.

ORDER

CRAVEN, Magistrate J.

***1** Pursuant to the provisions of 28 U.S.C. § 636(b)(1) and (3) and the Amended Order for the Adoption of Local Rules for Assignment of Duties to United States Magistrate Judges, Defendants' Motion to Stay (Docket Entry # 120) was referred to the Honorable Caroline M. Craven for the purposes of hearing and determining said motion. The Court, having considered the motion, response, and all relevant pleadings, is of the opinion the motion should be GRANTED.

I.

BACKGROUND

Plaintiff EchoStar Technologies Corporation ("Plaintiff") brings this cause of action against Defendants TiVo, Inc. ("TiVo") and Humax USA, Inc. ("Humax") (collectively "Defendants") alleging infringement, contributory infringement and/or inducement to infringe U.S. Patent No. 5,774,186 ("the '186 patent"), U.S. Patent No. 6,529,685 ("the '685 patent"), and U.S. Patent No. 6,208,804 ("the '804 patent"). Defendants generally deny these allegations and assert various affirmative defenses, which include non-infringement, invalidity and inequitable conduct. Additionally, Defendants assert counterclaims against Plaintiff for a declaratory judgment of non-infringement and invalidity of the patents-in-suit.

In the present matter before the Court, Defendants move the Court for a stay of this action in light of patent reexamination proceedings before the United States Patent & Trademark Office ("PTO"). According to Defendants, on May 25 and 26, and June 9, 2006, they filed requests for reexamination asking the PTO to reexamine and assess the patentability of all asserted claims of the three patents-in-suit. Defendants have requested an *ex partes* reexamination of the '186 and '804 patents and an *inter partes* reexamination of the '685 patent. Defendants assert substantial time and resources will be saved by staying the current proceedings in light of reexamination by the PTO.

II.

APPLICABLE LAW

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

"The district court has the inherent power to control its own docket, including the power to stay proceedings." *Soverain Sofnvare LLC v. Amazon.Com,* 356 F.Supp.2d 660, 662 (E.D.Tex.2005). "The power to stay proceedings is incidental to the power inherent in every court to control the disposition of the causes on its docket with economy of time and effort for itself, for counsel, and for litigants." *Landis v. North American Co.,* 299 U.S. 248, 254, 57 S.Ct. 163, 81 L.Ed. 153 (1936). "How this can best be done calls for the exercise of judgment, which must weigh competing interests and maintain an even balance." *Id.* at 254-55. In deciding whether to stay litigation pending reexamination, courts typically consider: "(1) whether a stay will unduly prejudice or present a clear tactical disadvantage to the nonmoving party; (2) whether a stay will simplify the issues in question and trial of the case; and (3) whether discovery is complete and whether a trial date has been set." *Soverain,* 356 F.Supp.2d at 662. Essentially, courts determine whether the benefits of a stay outweigh the inherent costs based on these factors.

***2** Additionally, a stay has been found to benefit the district court proceedings upon the completion of a reexamination:

> 1. All prior art presented to the Court will have been first considered by the PTO, with its particular expertise[;]
>
> 2. Many discovery problems relating to prior art can be alleviated by the PTO examination[;]
>
> 3. In those cases resulting in effective invalidity of the patent, the suit will likely be dismissed[;]
>
> 4. The outcome of the reexamination may encourage a settlement without the further use of the Court[;]
>
> 5. The record of reexamination would likely be entered at trial, thereby reducing the complexity and length of the litigation[;]
>
> 6. Issues, defenses, and evidence will be more easily limited in pretrial conferences after a reexamination[; and]
>
> 7. The cost will likely be reduced both for the parties and the Court.

Fisher Controls Co., 443 F.Supp. at 582 (S.D.Iowa 1977); *accord Emhart Industries v. Sankyo Seiki Mfg.,* 3 U.S.P.Q.2d 1889, 1890, 1987 WL 6314 (N.D.Ill.1987); *GPAC, Inc. v. D.W.W. Enterprises, Inc.,* 144 F.R.D. 60, 63 (D.N.J.1992). As noted by the Federal Circuit, reexamination may result in the elimination of most, if not all, of the issues remaining in the pending litigation. *See Gould,* 705 F.2d 1340 (Fed.Cir.1983), *cert. denied* 464 U.S. 935, 104 S.Ct. 343, 78 L.Ed.2d 310 (1983). If not found invalid, the reexamination will at least likely result in a narrowing and simplifying of the issues before the Court. *See Loffland Bros. Co. v. Mid-Western Energy Corp.,* 225 U.S.P.Q. 886, 887, 1985 WL 1483, at *2 (W.D.Okla. Jan.3, 1985). In addition, the technical expertise provided by the reexamination proceeding will be helpful to the Court on any issues that remain. *See Gould,* 705 F.2d at 1342.

III.

DISCUSSION

This case is the second patent infringement case concerning DVR technology to come before this Court involving the same parties. The first case, *TiVo, Inc. v. EchoStar Communications, et. al.,* C.A. No. 2:04-CV-1, was an infringement action in which Defendent TiVo sued Plaintiff for allegedly infringing on its patents covering DVR technology. That matter was tried before a jury and resulted in a verdict in favor of the plaintiff, Defendant TiVo. Notwithstanding, many issues remain unresolved in that case. To this end, Plaintiff asserts that "[a] stay would put [it] at a *huge tactical disadvantage* in the context of the overall litigation between the parties, and there is no just reason why this Court should not adjudicate [Plaintiff's] claims as expeditiously

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

as it did [Defendant's].” Plaintiff's Response at 4 (emphasis original). In addition, Plaintiff contends that a stay is not warranted based on the following: 1) Defendants failed to filed their requests for reexamination in a timely fashion; 2) the possibility of issue simplification is remote; and 3) discovery in this matter is near completion. Moreover, Plaintiff asserts that granting a stay is contrary to the law in this District.

***3** While recognizing the merits of Plaintiff's position, on balance, the Court finds that the equities weigh in favor of staying this matter pending reexamination. First, the Court is not persuaded that a stay would unduly prejudice Plaintiff. Although Plaintiff correctly notes that Defendants have not acted with dispatch in seeking reexamination and that Plaintiff has undoubtedly pursued an extremely burdensome discovery program, Plaintiff cannot say that its future costs associated with this litigation will be affected by the grant or denial of a stay. Further, the Court does not weigh in on the tactical effects on separate litigation. This matter stands on its own. Moreover, Plaintiff's argument is replete with one-sided supposition; it fails to consider the potential positive effects of a stay. To this end, Plaintiff's “tactical disadvantage argument” is without merit. The Court finds that staying this matter presents no clear tactical advantage for either party.

Further, Plaintiff fails to consider the potential effect of Defendants' *inter partes* reexamination request. The statute governing *inter partes* reexamination provides for full participation by a third party at all stages of the proceedings. *See* 35 U.S.C. § 311, *et. seq.* Unlike an *ex partes* reexamination, an *inter partes* reexamination allows the third-party requester “to file written comments addressing issues raised by the action of the Office or the patent owner's response thereto[.]” *Id.* at § 314(b)(2). In addition, the third-party requester may appeal to the Patent Board of Appeals and may appeal from the Board's decision to the Federal Circuit if the Board affirms a finding of patentability or reverses an examiner's finding of unpatentability. *Id.* at § 315(b)(1). Moreover, the third-party requester may participate as a party if the patent owner appeals to the court from an unfavorable decision regarding patentability. *Id.* at § 315(b)(2).

However, and of particular import here, the statute imposes estoppel restraints on a third-party requester. That is, a third-party requester is estopped from relitigating the same issue “which the third-party requester raised or could have raised during the *inter partes* reexamination proceedings.” *Id.* § 315(c); *see also Middleton, Inc. v. Minnesota Mining and Mfg. Co.*, 2004 WL 1968669, *10 (S.D.Iowa, 2004). In addition, the third-party requester will be estopped from seeking review of factual determinations made in the *inter partes* reexamination. *Id.* Thus, an *inter partes* reexamination can have no other effect but to streamline ongoing litigation. For these reasons, courts have an even more compelling reason to grant a stay when an *inter partes* reexamination is proceeding with the same parties, which is precisely the case here.

In addition, if the parties continue to litigate the validity of the claims in this Court, and the PTO subsequently finds that some or all of the claims in issue here are invalid, the Court will have wasted time and the parties will have spent additional funds addressing an invalid claim or claims. Thus, the grant of a stay will maximize the likelihood that neither the Court nor the parties expend their assets addressing invalid claims. And to the extent that claims survive the *inter partes* reexamination, Defendants will be precluded from challenging their validity in the same regard.

***4** Second, although there has been a great deal of activity in this litigation to date, much remains to be done before the case is ready for trial. Discovery is not yet completed, summary judgment motions have not been filed, and the Court has not completed its claim construction. It would be an egregious waste of both the parties' and the Court's resources if the *Markman* and summary judgment proceedings went forward and the claims were sub-

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

sequently declared invalid or were amended as a result of the reexamination proceeding.

Third, a stay will simplify the issues. As previously alluded to, the Court finds this issue is the predominate consideration in this case. Having the benefit of a *Markman* hearing under its belt, the Court finds that reexamination in this case will greatly influence the proceedings going forward. If the reexamination proceeding invalidates or narrows a claim or claims, the issues at trial will be simplified. But more importantly in light of the asserted scope of the claims at issue in this case, to the extent the reexamination proceeding reaffirm the claims at issue, the Court will then have the benefit of the PTO's expert analysis of the prior art that allegedly invalidates or limits the claims. *See GPAC Inc.*, 144 F.R.D. at 63 (noting that the PTO "is in a better position to evaluate the [patent at issue]" and the prior art due to its "requisite technical acumen"); *United Sweetener USA, Inc. v. Nutrasweet Co.*, 766 F.Supp. 212, 217 (D.Del.1991) (underscoring the PTO's expertise in dealing with reexamination); *Loffland Bros.*, 225 U.S.P.Q. (BNA) 886, 887, 1985 WL 1483, at *2 (W.D.Okla.1985) (granting a stay and stating that the "expert view of the Patent Office examiner will certainly benefit this Court"); *see also Gould*, 705 F.2d at 1342 (Fed.Cir.1983) (noting that reexamination procedures provide the court with "the expert view of the PTO").

The Court is cognizant that a stay may cause considerable delay in a case set for trial in February 2007 and sensitive to Plaintiff's right to have its day in court. Nevertheless, for the reasons previously expressed, the Court is convinced that a stay is appropriate in this particular case. Further, the Court reminds Plaintiff that each motion to stay pending reexamination filed in this Court is considered on a case by case basis with each cause of action presenting distinct circumstances; there exists no policy or rule in this District to "routinely" deny such motions. What is more, this case presents an issue which distinguishes it from those cases cited by Plaintiff in that Defendants have requested an *inter parties* reexamination of one the patents at issue in this case. As the Court has pointed out, this will have a dramatic effect on future litigation. Lastly, the Court notes that if, after reexamination, Plaintiff's patents are again upheld, Plaintiff's rights will only be strengthened, as the challenger's burden of proof becomes more difficult to sustain. *See Custom Accessories, Inc. v. Jeffrey-Allan Indus., Inc.*, 807 F.2d 955, 961 (Fed.Cir.1986) (holding that upon reissue, the burden of proving invalidity is "made heavier") (quoting *Interconnect Planning Corp. v. Feil*, 774 F.2d 1132, 1139 (Fed.Cir.1985)). To this end, and given the particular circumstances of this case, the court cannot find any undue prejudice to Plaintiff.

IV.

CONCLUSION

***5** Based on the foregoing, the Court finds that Defendants' motion to stay these proceedings pending reexamination of the patents-in-suit (Docket Entry # 120) should be granted. The Court finds a high likelihood that results of the PTO's reexamination will have a dramatic effect on the issues before the Court, up to and including dismissal of the entire action if the patent claims are found to be unpatentable. In any event, the Court will benefit from the PTO's expertise and determination on reexamination, and Plaintiff will not be unduly prejudiced by the stay. Therefore, it is hereby

ORDERED that Defendants' Motion to Stay (Docket Entry # 120) is GRANTED. It is further

ORDERED that the Clerk of the Court shall remove this matter from the active docket of the Court until the conclusion of the PTO's reexamination proceeding, and Defendants shall promptly advise the Court of the conclusion of the reexamination proceedings. It is further

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

ORDERED that all pending motion not addressed herein are DENIED WITHOUT PREJUDICE subject to refiling.

E.D.Tex.,2006.
EchoStar Technologies Corp. v. TiVo, Inc.
Not Reported in F.Supp.2d, 2006 WL 2501494 (E.D.Tex.)

END OF DOCUMENT

© 2010 Thomson Reuters. No Claim to Orig. US Gov. Works.

EXHIBIT 19

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

LIFE TECHNOLOGIES CORPORATION; APPLIED BIOSYSTEMS, LLC; INSTITUTE FOR PROTEIN RESEARCH; ALEXANDER CHETVERIN; HELENA CHETVERINA; and WILLIAM HONE,	)	
Plaintiffs,	)	C.A. No. 09-706 (RK)
v.	)	
ILLUMINA, INC. and SOLEXA, INC.,	)	
Defendants.	)	

PLAINTIFFS' FIRST SUPPLEMENTAL RESPONSES AND OBJECTIONS TO ILLUMINA AND SOLEXA'S INTERROGATORY NUMBERS 2, 3, 8, and 10

Pursuant to Rules 26 and 33 of the Federal Rules of Civil Procedure, plaintiffs Life Technologies Corporation ("LIFE") and Applied Biosystems, LLC ("AB") (collectively, the "Plaintiffs"), by and through their undersigned counsel, hereby object and supplement their responses to defendants Illumina, Inc. and Solexa, Inc. (collectively, the "Defendants") Interrogatory Nos. 2, 3, 8, and 10 to Plaintiffs.

These objections and responses represent Plaintiffs' reasonable efforts to provide the information requested based upon the information in their possession, custody, or control, and based upon their current knowledge and understanding. Plaintiffs' investigations are ongoing, and Plaintiffs reserve the right to alter or amend their objections and responses as set forth herein and to assert factual and legal contentions as additional facts are ascertained and analyses are made.

Confidential information disclosed in response to these Interrogatories may only be used in accordance to the Protective Order that will be jointly filed by the parties as agreed upon in Paragraph 5(c) of the Scheduling Order entered by the Court, dated October 30, 2009.

GENERAL OBJECTIONS

The Plaintiffs expressly incorporate their General and Specific Objections set forth in Plaintiffs Response to Defendants First and Second Sets of Interrogatories as if fully set forth herein.

SPECIFIC OBJECTIONS AND RESPONSES

INTERROGATORY NO. 1:

Separately, in claim-chart form and on a claim-by-claim and element-by-element basis for each claim of the Illumina Patents-In-Suit that You contend is invalid, describe: the legal and factual grounds on which You contend that each such claim is invalid, including; each document, testimony, prior knowledge, public use, test, experiment, and data on which You will rely in support of each such contention; to the extent You contend any claim is invalid under 35 U.S.C. §§ 102 or 103, where and how You contend each claim element is disclosed in the prior art, including specific references to pages, claims, columns and/or line numbers (if applicable) in each document supporting such contention; to the extent you contend any claim is invalid under § 102(a), the earliest date by which You contend the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country; to the extent You contend any claim is invalid under § 102(g), the alleged dates of conception and reduction to practice of the alleged prior art, and Your basis for contending that the alleged prior art was not abandoned, suppressed or concealed, and was diligently reduced to practice; to the extent You contend any claim is invalid under § 103, why the claim would have been obvious, including where the motivation to combine prior-art disclosures may be found and the level of skill of a person having ordinary skill in the art to which the subject matter of the Illumina Patents-In-Suit pertains at the time of the claimed inventions; to the extent You contend any claim is invalid under 35 U.S.C. § 112, how one of ordinary skill in the art would have purportedly found the claim indefinite, lacking an enabling disclosure, lacking sufficient written description, or lacking any contemplated best mode; each document, electronically stored information and thing that You rely on in support of Your answer; each person, other than counsel, who furnished information or was consulted regarding Your answer (including the nature and substance of each such person's knowledge and information); and the three individuals affiliated with You, other than counsel, most knowledgeable regarding the subject matter of this interrogatory (including the nature and substance of each such person's knowledge and information).

OBJECTION AND RESPONSE TO INTERROGATORY NO. 1:

In addition to their General Objections, Plaintiffs object to this Interrogatory to the extent it is not reasonably calculated to lead to the discovery of admissible evidence, vague and ambiguous (for example, in its use of the terms "most knowledgeable," "rely in support,"

and "furnished information or was consulted"), and overly broad and unduly burdensome (for example, in its use of the terms "each document, electronically stored information and thing" and "each person"). Plaintiffs also object to this Interrogatory as compound on the grounds that it constitutes multiple, discrete interrogatories by, for example, requesting information in multiple subparts on the two separate topics of invalidity and the level of ordinary skill in the art. Plaintiffs also object to this Interrogatory to the extent that it seeks legal conclusions and expert discovery. Plaintiffs further object to this Interrogatory to the extent that it seeks information protected by the attorney-client privilege or work product doctrine, or is otherwise immune from discovery. Finally, Plaintiffs object to this Interrogatory as premature, as Defendants have not yet specified the claims they are asserting.

Subject to, and without waiving their General and Specific Objections set forth above, Plaintiffs respond that the following prior art references render one or more claims of the Illumina Patents-In-Suit invalid under §§ 102 and/or 103:

- **U.S. Patent No. 6,831,994:** WO 96/12014; U.S. Pat. No. 6,151,405.
- **U.S. Patent No. 6,654,505:** WO 96/12014; J. Dow, *A Simple Microcomputer-Based System for Real-Time Analysis of Cell Behaviour*, 87 J. Cell Science 171-82 (1987).
- **U.S. Patent No. 7,232,656:** U.S. Patent No. 7,270,951; U.S. App. No. 09/266,187; M. Chee, et al., *Accessing Genetic Information with High-Density DNA Arrays*, 274 Science 610-14 (1996); U.S. Patent No. 5,202,231.
- **U.S. Patent No. 7,598,035:** U.S. Patent No. 5,677,153; W. Mandecki & T. J. Bolling, Fok*I Method of Gene Synthesis*, 68 Gene 101-07 (1988).

This investigation is not yet complete, and accordingly, Plaintiffs reserve their right to revise or supplement their response pursuant to Fed. R. Civ. P. 26(e), including the right to produce and identify documents under Fed. R. Civ. P. 33(d), as well as to accommodate Defendants' reasonable needs without undue burden.

FIRST SUPPLEMENTAL RESPONSE TO INTERROGATORY NO. 2:

Plaintiffs reassert their general and specific objections to Interrogatory No. 2, and supplement their previous response as follows. The Court recently revised the *Markman* scheduling deadlines in this case (D.I. 41). Accordingly, the Plaintiffs reserve the right to revise their response in light of any additional patent claims that the Defendants may assert against the Plaintiffs. Furthermore, the Plaintiffs reserve the right to supplement this Interrogatory response in light of the claim construction rendered by the Court, and in light of information revealed during fact discovery, which is still in its early stages.

One of skill in the art in the fields of the inventions disclosed in the Illumina Patents-In-Suit would be a person with graduate level training or post-secondary teaching experience in cellular biology, molecular biology, molecular genetics, or an equivalent field, and would have experience with techniques in this field including DNA sequencing and microscopy.

The following prior art references render one or more claims of the Illumina Patents-in-Suit invalid under §§ 102 and/or 103:

U.S. Patent No. 6,831,994:

1. Intl. Publ. No. WO 96/12014
2. U.S. Patent No. 6,057,150
3. Schmidt *et al.* Integrin-Cytoskeletal Interactions in Migrating Fibroblasts are Dynamic, Asymmetric, and Regulated. J. Cell Biology, 123(4): 997-991 (1993)
4. Gelles *et al.* Tracking kinesin-driven movements with nanometer-scale precision. Nature, 331: 450-453 (1988)
5. Wilson *et al.* Single particle tracking of cell-surface HLA-DR molecules using R-phycoerythrin labeled monoclonal antibodies and fluorescent digital imaging. J. Cell Science, 109: 2101-2109 (1996)
6. Dow *et al.* A simple microcomputer-based system for real-time analysis of cell behaviour. J. Cell Science, 87: 171-182 (1987)
7. NIH Image Version 1.58 Manual
8. U.S. Patent No. 6,151,405
9. U.S. Patent No. 5,415,839

10. Wernet and Pline. Particle displacement tracking technique and Cramer-Rao lower bound error in centroid estimates from CCD imagery. Experiments in Fluids, 15: 295-307 (1993)
11. U.S. Patent No. 5,556,764
12. Intl. Publ. No. WO 97/43611
13. DiMilla *et al.* Maximal Migration of Human Smooth Muscle Cells on Fibronectin and Type IV Collagen Occurs at an Intermediate Attachment Strength. J. Cell Biology, 122(3): 729-737 (1993)
14. U.S. Patent No. 5,257,182
15. U.S. Patent No. 6,122,396
16. Brandiss and Margel. Synthesis and Characterization of Self-Assembled Hydrophobic Monolayer Coating on Silica Colloids. Langmuir, 9: 1232-1240 (1993)

U.S. Patent No. 6,654,505:

1. Intl. Publ. No. WO 96/12014
2. Gelles *et al.* Tracking kinesin-driven movements with nanometer-scale precision. Nature, 331: 450-453 (1988)
3. U.S. Patent No. 6,057,150
4. Schmidt *et al.* Integrin-Cytoskeletal Interactions in Migrating Fibroblasts are Dynamic, Asymmetric, and Regulated. J. Cell Biology, 123(4): 977-991 (1993)
5. Wilson *et al.* Single particle tracking of cell-surface HLA-DR molecules using R-phycoerythrin labeled monoclonal antibodies and fluorescent digital imaging. J. Cell Science, 109: 2101-2109 (1996)
6. Dow *et al.* A simple microcomputer-based system for real-time analysis of cell behaviour. J. Cell Science, 87: 171-182 (1987)
7. NIH Image Version 1.58 Manual
8. Wernet and Pline. Particle displacement tracking technique and Cramer-Rao lower bound error in centroid estimates from CCD imagery. Experiments in Fluids, 15: 295-307 (1993)
9. U.S. Patent No. 5,415,839
10. U.S. Patent No. 5,599,668
11. U.S. Patent No. 5,556,764
12. Intl. Publ. No. WO 97/43611
13. U.S. Patent No. 6,151,405
14. DiMilla *et al.* Maximal Migration of Human Smooth Muscle Cells on Fibronectin and Type IV collagen Occurs at an Intermediate Attachment Strength. J. Cell Biology, 122(3): 729-737 (1993)
15. U.S. Patent No. 5,257,182
16. U.S. Patent No. 6,122,396

U.S. Patent No. 7,232,656:

1. Chee *et al.* Accessing Genetic Information with High-Density DNA Arrays. Science, 274:610-614 (1996) ("Chee I")
2. U.S. Patent No. 7,144,699 ("Chee II")

3. Intl. Publ. No. WO 96/12014 ("Brenner")
4. Weber and Meyers. "Human Whole-Genome Shotgun Sequencing" Genome Research, 7:401-409 (1997) ("Weber")
5. European Publ. No. EP 0 640 146 ("Rosenthal")
6. U.S. Patent No. 5, 302,509 ("Cheeseman")
7. Intl. Publ. No. WO 95/09248 ("Drmanac")
8. U.S. Pat. No. 7,270,951 ("Stemple")
9. Gunderson *et al.* Mutation Detection by Ligation to Complete *n*-mer DNA Arrays. Genome Research, 8:1142-1153 (1998) ("Gunderson")
10. Bentley. Decoding the Human Genome Sequence. Human Molecular Genetics, 9:2353-2358 (2000) ("Bentley")
11. Alderborn *et al.* Determination of Single-Nucloetide Polymorphisms by Real-Time Pyrophosphate DNA Sequencing. Genome Research, 10:1249-1258 (2000) ("Alderborn")
12. Bentley. The Human Genome Project – An Overview. John Wiley & Sons, Inc. (2000) ("Bentley II")
13. Delcher *et al.* Alignment of Whole Genomes. Nucleic Acid Research, 27:2369-2376 (1999) ("Delcher")
14. Altshuler *et al.* An SNP Map of the Human Genome Generated by Reduced Representation Shotgun Sequencing. Nature, 407:513-516 (2000) ("Altshuler")

U.S. Patent No. 7,598,035:

1. Mandecki and Bolling, "*Fok*I method of gene synthesis" Gene 68:101-107 (1988) ("Mandecki")
2. Szybalski *et al.*, "Class-IIS restriction enzymes – a review" Gene 100:13-26 (1991) ("Szybalski")
3. U.S. Patent No. 5,827,704 ("Cease I")
4. Cease and Lohff, "A vector for facile PCR product cloning and modification generating any desired 4-base 5' overhang: pRPM" BioTechniques 14:250-255 (1993) ("Cease II")
5. Dobrynin *et al.*, "Plasmid vectors pBBV for cloning and regeneration of DNA fragments with any end nucleotide sequence" Reports of the USSR Academy of Sciences 278:1002-1005 (1984) ("Dobrynin")
6. Kuznedelov *et al.*, "Plasmid vector pSSCI for cloning synthetic polynucleotides and their regeneration with a unique terminal nucleotide sequence" Bioorganic Chemistry 12:842-844 (1986) ("Kuznedelov")
7. Palzkill and Botstein, "Probing β-lactamase structure and function using random replacement mutagenesis" Proteins 14:29-44 (1992) ("Palzkill")
8. U.S. Patent No. 5,677,153 ("Botstein")
9. Mormeneo *et al.*, "Precise nucleotide sequence modifications with bidirectionally cleaving class-IIS excision linkers" Gene 61:21-30 (1987) ("Mormeneo")
10. Poustka *et al.*, "Construction and use of human chromosome jumping libraries from *Not*I-digested DNA" Nature 325:353-355 (1987) ("Poustka I")
11. Poustka and Lehrach, "Jumping libraries and linking libraries: the next generation of molecular tools in mammalian genetics" Trends in Genetics 2:174-179 (1986) ("Poustka II")

12. Sapolsky and Lipshutz, "Mapping genomic library clones using oligonucleotide arrays" Genomics 33:445-456 (1996) ("Sapolsky I")
13. U.S. Patent No. 5,710,000 ("Sapolsky II")
14. Velculescu *et al.*, "Serial analysis of gene expression" Science 270:484-487 (1995) ("Velculescu")

The Plaintiffs have prepared claim charts that pair the limitations of the claims of the Illumina Patents-In-Suit with their corresponding disclosures in the prior art. The pairing of the prior art with the claims of the Illumina Patents-In-Suit is based upon the Plaintiffs' current assumptions regarding the claim construction positions necessitated by Defendants' infringement contentions. The charts for each patent are broken into separate exhibits as set forth below:

Exhibit A: U.S. Patent No. 6,654,505
Exhibit B: U.S. Patent No. 6,831,994
Exhibit C: U.S. Patent No. 7,232,656
Exhibit D: U.S. Patent No. 7,598,035

The claims of the following patents are also invalid under 35 U.S.C. § 112 for at least each of the following reasons:

U.S. Patent No. 6,831,994: In claim 1, "optical train effective to record" is not described or enabled.

U.S. Patent No. 7,232,656: In claim 2, "genetic signature" is indefinite and not described or enabled; in claim 4, "said solid support" and "each molecule" lack antecedent basis; "known genomic reference sequence" was not enabled; and all claims are not adequately described.

U.S. Patent No. 7,598,035: In claim 3, "one of said pair of segments is located at one end of each of the linearized vectors, and another of said pair of segments is located at another end of each of the linearized vectors" is indefinite and not described or enabled.

INTERROGATORY NO. 3:

Describe the circumstances under which You first became aware of each of the Illumina Patents-In-Suit, including the person(s) employed by or associated with You who first became aware of each of the Illumina Patents-In-Suit; the date(s) on which such person(s) first became aware of each of the Illumina Patents-In-Suit; the step(s) taken to investigate whether

Illumina's nucleic acid amplification and sequencing products and a comparison of that information with the claims of the Chetverin patents-in-suit. This publicly available information included information published on Illumina's website and in *Nature*, such as Bentley et al., "Accurate whole human genome sequencing using reversible terminator chemistry," 456 *Nature* 53-59 (2008).

This investigation is ongoing, and accordingly, Plaintiffs reserve their right to revise or supplement their response pursuant to Fed. R. Civ. P. 26(e), including the right to produce and identify documents under Fed. R. Civ. P. 33(d).

MORRIS, NICHOLS, ARSHT & TUNNELL LLP

/s/ Jack B. Blumenfeld

Jack B. Blumenfeld (#1014)
Maryellen Noreika (#3208)
1201 North Market Street
P.O. Box 1347
Wilmington, DE 19899
(302) 658-9200
jblumenfeld@mnat.com
mnoreika@mnat.com

Attorneys for Plaintiffs

OF COUNSEL:

Nicholas Groombridge
Peter Sandel
Jenny Wu
WEIL, GOTSHAL & MANGES LLP
767 Fifth Avenue
New York, NY 10153
(212) 310-8242

February 26, 2010

CERTIFICATE OF SERVICE

I hereby certify that copies of the foregoing were caused to be served on February 26, 2010 upon the following in the manner indicated:

VIA ELECTRONIC MAIL

Steven J. Balick, Esquire
Lauren E. Maguire, Esquire
Andrew D. Cordo, Esquire
ASHBY & GEDDES
500 Delaware Avenue – 8th Floor
Wilmington, DE 19801

Kevin M. Flowers, Esquire
Matthew C. Nielsen, Esquire
Mark H. Izraelewicz, Esquire
John R. Labbé, Esquire
Cullen N. Pendleton, Esquire
MARSHALL, GERSTEIN & BORUN LLP
233 South Wacker Drive, Suite 6300
Chicago, IL 60606

/s/ Jack B. Blumenfeld

Jack B. Blumenfeld (#1014)

EXHIBIT 20

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Inter Partes Reexamination Filing Data – December 31, 2009

1. Total requests filed since start of *inter partes* reexam on 11/29/99 ..... 808[1]

2. Number of filings by discipline

a.	Chemical Operation	158	20%
b.	Electrical Operation	385	48%
c.	Mechanical Operation	253	31%
d.	Design Patents	12	1%

3. Annual Reexam Filings

Fiscal Yr.	No.	Fiscal Yr.	No.	Fiscal Yr.	No.	Fiscal Yr.	No.
2000	0	2003	21	2006	70	2009	258
2001	1	2004	27	2007	126	2010YTD	74
2002	4	2005	59	2008	168		

4. Number known to be in litigation……...…….…….……..552……...68%

5. Decisions on requests 699

   a. No. granted ..... 668 ..... 96%

      (1) By examiner 667
      (2) By Director (on petition) 1

   b. No. not granted ..... 31 ..... 4%

      (1) By examiner 27
      (2) Reexam vacated 4

6. Overall reexamination pendency (Filing date to certificate issue date)
   a. Average pendency 36.2 (mos.)
   b. Median pendency 31.9 (mos.)

7. Total inter partes reexamination certificates issued (1999 - present) 134

a.	Certificates with all claims confirmed	11	8%
b.	Certificates with all claims canceled (or disclaimed)	68	51%
c.	Certificates with claims changes	55	41%

[1]Of the requests received in FY 2010, 9 requests have not yet been accorded a filing date, and 2 requests have had preprocessing terminated, for failure to comply with the requirements of 37 CFR 1.915. See Clarification of Filing Date Requirements for *Ex Parte* and *Inter Partes* Reexamination Proceedings, Final Rule, 71 Fed. Reg. 44219 (August 4, 2006).