

EXHIBIT 2

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**IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE**

10X GENOMICS, INC. and PRESIDENT AND
FELLOWS OF HARVARD COLLEGE,

Plaintiffs,

v.

VIZGEN, INC.,

Defendant.

C.A. No. 22-595-MFK

**RESPONSIVE EXPERT REPORT OF RAHUL SATIJA, D. Phil.
REGARDING VALIDITY OF THE CHURCH PATENTS**

Dated: September 25, 2024

By:

A handwritten signature in black ink that reads "Rahul Satija". The signature is written in a cursive, flowing style.

Rahul Satija, D. Phil.

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3. “decoding reagent”

54. I understand that the Court found that “decoding reagent” should have its plain and ordinary meaning. Claim Construction Order at 17-20. I understand that the Court rejected Vizgen’s argument that “decoding reagent” means “any reagent that can yield a signal signature,” instead siding with 10x who argued that Vizgen’s construction could be confusing as “yield” a signal signature could be understood to exclude the absence of color as a valid signal signature. Claim Construction Order at 18-19. I have applied the Court’s construction in my analysis.

4. “analyte”

55. I understand that the Court found that “analyte” means “the molecule detected, identified, or measured by binding of a detection reagent whose probe reagent(s) recognize it (i.e., are specific partners thereto)” and rejected Vizgen’s proposed construction of “the molecule detected, identified, or measured by binding of a detection reagent whose probe reagent(s) recognize (i.e., are specific partners) thereto.” Claim Construction Order at 23-25. I understand that the court rejected Vizgen’s argument that the specification requires Vizgen’s construction, the Court noting the grammatical error is evident from the face of the patent; it is not subject to debate based on the claim language and specification; and the prosecution history does not suggest a different interpretation of the claims affected by that grammatical error. Claim Construction Order at 25. I have applied the Court’s construction in my analysis.

V. OVERVIEW OF THE ASSERTED SEQTAG PROBE PATENTS

A. Priority and Overview of the SeqTag Probe Patents

56. The asserted claims of the 737, 051, 052, and 136 Patents share a common specification and are all entitled, “Compositions and Methods For Analyte Detection.” The inventors of the SeqTag Probe patents are George Church, Je-Hyuk Lee, Daniel Levner, and

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Michael Super. The SeqTag Probe Patents teach methods for *in situ* multiplex analyte detection, where analytes are identified based on the temporal sequence of signal signatures.

57. I understand that the SeqTag Probe Patents claim priority to the following patent documents:

- Provisional application no. 61/777,383, filed on March 12, 2013 (the “383 provisional” or “3D matrix provisional”) (10XH-00004923);
- PCT application no. PCT/US2012/071398, filed on December 21, 2012 (the “PCT application”) (10XH-00004551);
- Provisional application no. 61/579,265, filed on December 22, 2011 (the “265 provisional” or “SeqTag Probe provisional”) (10XH-00004808).

58. [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

B. The Shared Specification of the SeqTag Probe Patents

1. Overview Of The Temporal Order Method

59. The SeqTag Probe patents are directed to methods of *in situ* multiplex analyte detection where analytes are detected based upon a temporal order of signal signatures. 051 Patent³, 3:62-4:1; 9:25-30; 20:44-54. The detection reagents and methods outlined in the SeqTag Probe Patents can be utilized in biological assays for detecting, identifying, and/or quantifying target molecules or analytes in a sample (8:19-22). The specification teaches methods which perform cycles of steps (3:62-4:1; 4:34-55; 5:15-17; 20:44-53), where each cycle results in detection of a distinguishable label (for example, a fluorescent color attached to a bound probe, or no color if a probe did not bind) (6:4-22; 27:33-37; 29:32-36; 37:37-67). The cycles are repeated,

³ All patent cites throughout refer to the 051 patent unless indicated otherwise.

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and the consecutive detection steps together yield a temporal sequence of signals signatures. This temporal sequence serves as a barcode that can identify a target analyte at a specific location within the sample. Refer to for example, 3:62-4:55; 10:44-12:7; 19:48-20:54; 70:16-76:24; 79:60-82:54.

60. The patent aims to address a key limitation of traditional *in situ* assays, where the number of analytes that can be simultaneously measured is sharply constrained by the availability of distinct optical labels. The patent explains that its objective was to “significantly increase the number of different probes (and corresponding analytes) that can be simultaneously detected in a multiplex assay, as compared to an traditional assay where each probe is labeled with only fluorescent labels or quantum dots, and thus multiplexing is limited by the number of available and practically usable colors.” 19:56-62; *see, e.g., id.*, 63:56-57 (“As with other fluorescence-based techniques, FISH is limited to the number of colors available to the microscopy.”).

61. The patent explains that for *in situ* detection, the limitation on the number of colors posed a significant drawback that could not be overcome with traditional methods. For example, while successive hybridization steps between probes and target analytes could in principle increase diversity, the patent describes how this approach would require “multiple lengthy probe incubations and damaging stripping steps.” 27:48-50. *See id.*, 63:37-42 (“While very common, immunohistochemistry is typically limited to probing with a small set of antibodies at a time (due to the limitation of available optical colors), and multiple staining cycles are generally avoided, since the stripping of the preceding cycle’s antibodies can damage the sample”); 63:66-64:3 (“Using some embodiments of the detection reagents and/or methods described herein, the assay can capture and probe numerous mRNA and/or miRNA at once, reducing and/or eliminating potentially harmful stripping steps.”).

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62. The patent describes multiple problems faced by prior art methods that sought to overcome the limited number of colors using alternative strategies. For example, the patent describes how the use of nanostring technology can utilize different ratios of colors to increase multiplexing (9:31-62), but highlights that the “technology generally requires very high optical magnification for spatially discerning separation of colors that are typically located very close to each other within a nanostring; thus limiting a field of view/sample size, and precision, and/or increasing instrument cost” (9:50-56). Second the “technology generally requires a thorough control of the amount of probes used in detection, because too few probes would yield a signal that is difficult to be detected, but too many probes would increase the likelihood of probes overlapping, and thus making the readout impossible” (9:57-62). The patent explains that these problems were caused by the simultaneous spatial location of signals, which was known to occur when multiple target analytes are closely located (“crowded”) together in a biological sample.

63. The patent explains how the temporal order detection method substantially increased multiplexing capacity: by performing a series of sequential hybridization steps and performing a detection step at each cycle, the set of distinct possible outputs generates substantial combinatorial diversity even when only a limited set of distinguishable optical labels are available: “because the detection reagents described herein are detected and/or imaged in a temporal series of steps, the number of probes (and corresponding analytes) that can be detected in a multiplex assay grows multiplicatively with the number of detection steps in a time series and the number of optical labels being used.” 19:62-20:1; *see id.*, 63:58-62 (“Using some embodiments of the detection reagents and/or methods described herein, the cells can be probed for many sequences simultaneously, thus allowing the user to save time and sample material, extract more data, and demand less prior knowledge of the sample.”).

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64. The specification describes how, in advance of detecting an optical signal, the claimed methods generally add a plurality of detection reagents to a sample. 051 Patent at 20:5-24; 70:16-38. Each detection reagent consists of a at least one probe reagent, which can contain a binding domain for hybridizing or specifically binding to a target analyte. The detection reagent also comprises a plurality of pre-determined nucleic acid subsequences which can be uniquely associated with the target analyte. 29:12-37; 37:20-22. After the detection reagents bind to the analytes, decoder probes or decoding reagents are added. 22:29-24:25; 24:52-26:10: The decoder probes are associated with a detectable label (i.e. a 'color') (71:8-11), and may be engineered to hybridize with the nucleic acid sequences on the detection reagent. 24:52-25:6, 68:1-67; 71:12-63. The sample is then imaged, and, if a decoder probe hybridizes, its associated signal will be detected. 25:11-19. If a decoder probe did not bind, then no color (or dark) will appear when imaged. (051 Patent at 6:4-12; 6:4-17; 6:9-17; 8:9-11; 23:16-18; 25:23-42; 26:11-18; 27:33-41; 29:32-36; 29:61-66; 37:55-67; 37:55-67; 38:7-14; 64:41-44). This general sequence of steps is repeated, generating a temporal sequence of optical signatures that can be used to decode the set of pre-determined subsequences associated with the detection reagent, thereby identifying the target analyte. Refer to for example, 28:58-29:11; 70:16-38. The specification describes these reagents and steps in detail.

2. Samples (including sample preparation and analytes)

65. The specification describes methods and techniques for *in situ* analysis, allowing for the detection of target analytes directly on samples that comprise cells and biological tissues. 4:15-26, 22:1-6, 34:60-35:14, 59:65-60:3, 60:16-41, 60:42-57, 67:16-30. The specification describes that the methods can be adapted for use in immunofluorescence or *in situ* hybridization (30:17-27), and describes steps to optionally prepare the sample for *in situ* analysis, even prior to contacting the sample with detection reagents (4:56-58, 8:37-44, 21:49-22:18, 34:36-45, 61:12-

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situ detection. Indeed, the patent distinguishes the claimed methods (which can be performed *in situ*), with prior art methods (which could not):

Accordingly, the compositions and the methods described in the '824 application cannot be used and detected directly on a sample (e.g., on a tissue sample) or *in situ* as described herein, e.g., immunofluorescence, immunohistochemistry, fluorescence *in situ* hybridization, or western blot.

051 Patent at 9:25-30.

133. What's more is that Dr. Metzker fails to acknowledge numerous passages in the specification describing in-situ analysis and fails to recognize that the inventors were describing the temporal detection method as enabling higher-plex *in situ* assays. A POSA would recognize that when the specification teaches the 'two-step' detection reagent—decoder probe approach that allows for in-situ analysis. In this process, the target molecule binds to a detection reagent in an initial step, and the identity of the detection reagent is subsequently decoded via sequential hybridizations of decoder probes with distinguishable labels. For example:

One aspect of the inventions provides the methods for detecting a plurality of analytes in a sample, using the detection reagents described herein. The method includes (a) contacting the sample with a composition comprising a plurality of detection reagents (which will be described in detail later), wherein each subpopulation of the detection reagents targets at least one different analyte; and (b) detecting in a temporally-sequential manner said plurality of the pre-determined subsequences of said detection reagents, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents.

051 Patent at 20:5-24.

1. Multiplexing

134. The patent describes that plexy of prior art *in situ* methods was limited because the number of available colors as detectable probe labels was limited to only three or four. 051 Patent at 63:56-57 ("As with other fluorescence-based techniques, FISH is limited to the number of colors

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available to the microscopy.”); *see* 9:31-62; 19:56-62; 63:37-42. The patent describes how multiplexing is increased by indirectly linking the decoder probe to the analyte and using temporal readout cycles with only a subset of analytes having a color readout each cycle.

The detection reagents and methods described herein significantly increase the number of different probes (and corresponding analytes) that can be simultaneously detected in a multiplex assay, as compared to an traditional assay where each probe is labeled with only fluorescent labels or quantum dots, and thus multiplexing is limited by the number of available and practically usable colors. Furthermore, because the detection reagents described herein are detected and/or imaged in a temporal series of steps, the number of probes (and corresponding analytes) that can be detected in a multiplex assay grows multiplicatively with the number of detection steps in a time series and the number of optical labels being used. By way of example only, 3 set of images in which 4 distinct optical labels are used can encode $4 \times 4 \times 4 = 64$ distinct probe reagents (e.g., antibodies).

051 Patent at 19:56-20:3; 37:37-67.

2. Optical crowding and Signal Amplification

135. Because detection is not limited to one detectable event but a barcode, the temporal method allows for most analytes to appear dark in any readout cycle. The patent describes that “no color” or “dark” is a signal signature. Refer to for example, 051 Patent at 29:32-34 (“signatures of fluorescent color, visible light, no-color, and any combinations thereof”).

136. A POSA would understand that when the patent describes the temporal detection method and the inclusion of the absence of color as a color, the specification is describing *in situ* detection because it is addressing previously known problems of *in situ* detection. For example, prior art methods suffered from “too many probes [that] would increase the likelihood of probes overlapping, and thus making the readout impossible.” 051 Patent at 9:61-62. Keeping most detection reagents dark during any particular readout cycle cleverly minimizes signal overlap from decoder probes that could overlap in 3D space when a tissue sample is imaged. A POSA would recognize that this approach was being described as particularly valuable in tissue samples, where the high density of molecules in particular regions of the cell results in the physical crowding of

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understand would limit potential spatial movement, and further alleviate this problem. Refer also to 26:19-43.

6. Presence or Absence of Working Examples

265. Dr. Metzker asserts there are no working examples in the specification because “the one experiment described in the specification relates to the identification of an extracellular analyte of a yeast cell, i.e., not in a cell and not in a tissue sample.” Metzker Rpt., ¶ 924. I disagree.

266. The specification clearly describes how a temporal signature can be used to identify detection reagents through sequential hybridization and detection of optical signals in a cell sample. The detection reagents comprise antibodies that are pathogen-specific probes and associated with distinct nucleic acid labels via conjugation to nanoparticles, consistent with the specification. The detection reagents are exposed to a sample of yeast cells, where they specifically bind to target pathogens. The identities of the detection reagents are then decoded via sequential hybridization steps with fluorescently labeled decoder probes, which emit a temporal signature. A POSA would recognize that this experiment represents a working example of the claimed invention.

267. Dr. Metzker argues that this example (Example 1) does not meet the requirements of the asserted claims because the probe reagent binds to the outside of a yeast cell, and does not enter the cell itself. A POSA would not agree with Dr. Metzker’s conclusion that an *in situ* experiment requires a probe reagent to enter an individual cell. Instead, a POSA would understand that the phrase “in a cell or tissue sample” requires the *in situ* analysis to be performed within a group of cells or a piece of biological tissue. A POSA would recognize that the example experiment provided in the specification is performed on a sample of yeast cells, and therefore demonstrates the claimed invention.

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more pre-determined subsequences; and

(c) using said temporal order of signal signatures to identify said analyte in said cell or tissue sample.

283. A POSA would understand with reasonable certainty that a temporal order can be associated with one predetermined subsequence due to the two-step detection approach. The patent contemplates that in any given detection readout round, not every detection reagent will have a decoder probe bind such so that round will register as dark. *See* Section V.B.4, VII.D, VII.E. So even with only one pre-determined subsequence, a temporal order can be created where multiple signal signatures are associated with the predetermined sequence over time, such as a combination of colored-light and dark cycles. The patent contemplates that the number of predetermined subsequences and colors impact the plexy of the system and a POSA would understand how to design the assay parameters accordingly. For example, refer to 37:37-67. Further, a POSA would understand that in these embodiments, a single pre-determined subsequence can be repeatedly profiled with different subpopulations of decoder probes with different detectable labels (including a ‘no color’ label), resulting in the generation of a temporal signature that can be used to identify the detection reagent and corresponding analyte.

284. Dr. Metzker opines that it is “unclear how an embodiment using one predetermined subsequence would differ from the prior art.” Metzker Rpt., ¶ 952. As explained above, the two step detection approach differs from the prior art in allowing the temporal detection of analytes. Dr. Metzker takes issue that the specification describes the temporal order as a way to increase multiplexing. As explained throughout, the temporal order does increase plexy but a POSA would know if she were interested in an assay with fewer targets, it can be adapted by changing the number of predetermined subsequences and/or colors. That does not render the claim term indefinite.

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any portions of the Levsky 2002 article (let alone any specifically identified portions) into the Gunderson 2004 article.

B. There Is a Lack of Motivation to Combine and Lack of Reasonable Expectation of Success for the Combinations Dr. Metzker Relies on for His Opinions Regarding Alleged Obviousness

343. Dr. Metzker's Report relies on attempts to combine references describing *in situ* analysis techniques (Larsson Dissertation, Larsson 2004, Larsson 2010, (collectively the Larsson references), Sood 043, and Levsky 2002 (collectively the *in situ* art)) with non-*in situ*, array-based techniques that were designed and demonstrated for use in the very different context of an artificially simplified and controlled ex situ array (Göransson 2009, Göransson Dissertation, and Gunderson 2004, collectively, the "array-based art").

344. A person of ordinary skill in the art at the time of the invention of the Church SeqTag Probe Patents in 2010-2011²² would not have been motivated to combine the specialized techniques of the *in situ* art with the specialized techniques of the array-based art or had any reasonable expectation for the combination to succeed. As I describe below, a person of ordinary skill in the art was aware of the many differences between the *in situ* environment and the much simpler and more controlled environment involved in the use of non-*in situ* array techniques, and a person of ordinary skill working on *in situ* techniques would not have thought to look to array based techniques or expected such techniques to be successful in the complex *in situ* context nor would a person of ordinary skill working on array-based techniques have expected their techniques to be applicable to (or successfully combine with) *in situ* detection techniques. Rather than being

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obvious to combine with each other, the techniques of the *in situ* art relied on by Dr. Metzker and the techniques of the array-based art relied on by Dr. Metzker each separately worked well for their respective purposes and one of ordinary skill would not have expected success from combining them due to their very different (and incompatible) techniques and contexts.

1. Lack of Motivation to Combine In Situ Detection Techniques with Array-Based Sequential Detection Techniques and Lack of Reasonable Expectation of Success From Such a Combination

345. There are unique technical challenges associated with performing sequential rounds of hybridization directly on biological tissue samples. In *in situ* approaches, the spatial relationships and native cellular environment are preserved, allowing researchers to study molecules in their natural setting, which is critical for understanding biological processes at the cellular and subcellular levels. By contrast, in an array, the molecules are isolated from their original context, immobilized on a solid surface, and arranged in a highly controlled manner that simplifies detection but removes the spatial and environmental factors that can influence biological interactions. This distinction is crucial, as it highlights the limitations of applying array-based methods directly to *in situ* studies. The immobilization and alignment benefits in arrays do not translate to *in situ* methods, where molecular movement and complex three-dimensional arrangements introduce variability that cannot be accounted for by techniques developed for *ex situ* environments. Dr. Metzker's failure to consider these fundamental differences undermines the applicability of array-based solutions to more complex *in situ* detection challenges.

346. Dr. Metzker fails to acknowledge fundamental differences between a biological tissue sample where target analytes are located *in situ* and an array used to analyze molecules that have been extracted and analyzed *ex situ*. In an array, the target molecules are isolated from their original cellular context, immobilized on a solid surface, and arranged in a highly controlled manner. This process represents an ideal set of conditions for sequential hybridization and image-

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based analysis, but that would not be applicable to in-situ analysis performed on a biological tissue sample.

347. First, array-based methods can control for the density of molecules on the solid surface by altering the distance between the probes. These methods can therefore ensure that distinct molecules are not too densely packed in any given region. In contrast, analytes in a biological tissue sample are identified in their original biological and cellular context and may be localized to dense regions where multiple distinct analytes are crowded together. This can result in the blending of optical signal originating from two distinct analytes that are crowded together, a phenomenon known as 'optical crowding'.

348. As previously mentioned, the issue of optical crowding becomes more problematic in highly multiplexed assays, but it is less of a concern in low-plex assays with fewer target analytes. Consequently, *in situ* analyses that detect a limited number of targets in a single round of hybridization did not face significant challenges from optical crowding. Similarly, array-based approaches with sequential hybridization steps could manage molecular density to avoid this issue. However, a person of ordinary skill in the art would recognize that attempting to combine these two techniques would appear fundamentally incompatible due to the inherent problem of optical crowding.

349. Second, array-based methods utilize detection reagents that are immobilized on a solid support, preventing their movement over the course of an experiment. The lack of movement between subsequent rounds helps to ensure that an optical signal emitted from a detection reagent during the first round of imaging will spatially co-localize with an optical signal emitted during any subsequent imaging round of the same detection reagent. Therefore, the artificial

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immobilization of detection reagents helps to ensure that the resulting optical signatures remain spatially aligned across multiple rounds of imaging.

350. Prior *in situ* art that utilized a single round of hybridization/detection/imaging did not require aligning optical signatures across multiple images. Since only a single imaging step was performed, potential movement of non-immobilized detection reagents or analytes did not represent a technical challenge. Array-based methods utilized multiple rounds of hybridization and detection/imaging but alignment was facilitated via immobilized detection reagents. However, a person of ordinary skill in the art would recognize that attempting to combine the two approaches would appear to be fundamentally incompatible as the detection reagents, and therefore the resulting optical signatures, may not be aligned across multiple imaging cycles.

351. Third, sequential hybridization methods require multiple rounds of hybridization, dehybridization, and visualization to generate an optical signature. When performing this experiment in-situ, the optical signal and intensity from each round of imaging will decrease. In particular, any decrease in the efficiency for each experimental step from one cycle to the next will result in an exponential decay over the course of the experiment. For example, biological analytes (especially particularly unstable molecules such as RNA) are likely to be degraded by the conditions required for dehybridization, such as exposure to high heat and/or treatment with harsh chemicals. This problem is minimized in array-based experiments, which benefit from pristine conditions, as well as the ability to precisely add and remove reagents from the array to optimize efficiency across multiple cycles. However, this problem is exacerbated during *in situ* analysis, where molecular reagents must contend with additional components of the biological sample that may restrict the movement of probes, nucleic acids, and chemicals and dampen optical signal. A skilled artisan would not be motivated to perform sequential rounds of hybridization *in situ* due to

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concerns about maintaining the efficiency and signal strength of the hybridization and detection process across multiple cycles.

352. Fourth, imaging experiments performed *in situ* are subject to autofluorescence, the natural emission of light by biological structures during imaging experiments. Many components of a biological tissue sample including structural proteins and the extracellular matrix can cause autofluorescence.²³ This phenomenon reduces the signal-to-noise ratio for bona fide signal originating from fluorescent molecules, and therefore can complicate fluorescence microscopy experiments in immunohistochemistry or *in situ* hybridization workflows. This problem can be exacerbated during sequential cycles of imaging, as the cumulative degree of autofluorescence can increase over the duration of the experiment. For example, fluorophores can attach or otherwise associate non-specifically to background components in the cell or tissue sample and as multiple rounds of fluorophores are applied, more and more attach and the background autofluorescence problem worsens. As a result, the signal-to-noise ratio can decay exponentially over multiple rounds of imaging. In contrast, array-based workflows mitigate this technical challenge as they take place in pristine and controlled environments that lack extraneous biological molecules that can cause autofluorescence.

353. Fifth, probe accessibility in *in situ* experiments is inherently limited by the three-dimensional nature of biological tissues. In contrast to arrays, where probes are freely accessible and applied in controlled conditions on a flat, two-dimensional surface, biological tissues present

²³ This is confirmed by, for example, the 2011 Itzkovitz Review article that I discuss elsewhere in this Report. Itzkovitz and van Oudenaarden, Validating Transcripts with Probes and Imaging Technology, Nat Methods 8(4 Supp) (Itzkovitz 2011) at S16 (“Image signal is often masked by noise stemming from both instrument-related factors such as dark current, pixelation noise and CCD readout noise **but mostly from out-of-focus light from cellular autofluorescence.** . . . Excitation filters can narrow this band to the fluorophore excitation wavelength, **but autofluorescence always remains.**” (HARV0106325-332 at 329.)

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structural challenges. Dense or complex tissue regions may inhibit efficient probe penetration, especially over multiple cycles of hybridization and dehybridization. A person of ordinary skill in the art would recognize that this difference creates a significant barrier to the successful application of sequential hybridization *in situ*, as uneven probe access can lead to incomplete or inconsistent labeling of target analytes, further compounding optical alignment and signal decay issues.

354. Dr. Metzker fails to acknowledge these technical challenges in his analysis. However, a person of ordinary skill in the art would be aware of these incompatibilities that would create significant challenges when attempting to combine the *in situ* and array-based art. A person of ordinary skill in the art who was interested in successfully increasing the plexity of *in situ* analyses would therefore not be motivated to pursue sequential hybridization approaches, and would instead look for alternative techniques.

355. As I have previously discussed, this is exactly how researchers in the field approached the challenge of increasing multiplexity prior to the Church SeqTag Probe Patents. Active researchers in the field, including Long Cai and Robert Singer, all were deeply interested in increasing multiplexing capacity and proposed a variety of strategies to do so. Prior to the Church SeqTag Probe Patents, these proposed techniques leveraged strategies to increase the number of detectable labels in a single round of imaging. These strategies were diverse and included utilizing multiply labeled probes as a detectable label, measuring the ratio of colors, replacing traditional fluorophores with quantum dots, and measuring a spatially ordered signature. However, these proposed methods did not suggest the use of sequential hybridizations or multiple rounds of detection to generate a temporal order of signal signatures. I discuss this further in the section of this Report regarding Objective Indicia of Non-Obviousness, below.

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356. I also disagree with Dr. Metzker's suggestion that once an array-based technique or other such technique that is designed for detection of biological targets outside of cells or tissue samples is shown to be successful in its own unique array-based or other *ex situ* environment, the next logical step is to attempt to apply such a technique to *in situ* detection. That assertion is contrary to all of the *in situ* detection references cited both in my report and in Dr. Metzker's report. Because of the many complexities I describe above that are involved in *in situ* detection, successful adoption of techniques that work outside of cells or tissue for detection *in situ* is a rare exception and certainly not a common or "next logical" step.

357. Dr. Metzker's suggestion is contradicted by examples of molecular biology techniques that have been successfully and routinely utilized in ex-situ assays, but have never been successfully performed *in situ* despite substantial interest. For example, Sanger sequencing is a widely used ex-situ technique that has been highly successful in sequencing DNA extracted from cells or tissues. Sanger sequencing synthesizes DNA using chain-terminating nucleotides that stop DNA synthesis at different bases, thereby creating fragments of varying lengths. These fragments are then separated by size to determine the DNA sequence. Its invention by Frederick Sanger in 1977 enabled researchers to read the genetic code stored in DNA molecules, revolutionized molecular biology and genetics, and won the Nobel Prize in Chemistry in 1980.

358. Since its invention, there has been extensive interest in extending Sanger Sequencing to *in situ* assays, which would enable researchers to directly read the sequence of DNA molecules in biological cell and tissue samples. However, Sanger Sequencing has never been successfully applied *in situ*. This is due to extensive technical challenges for performing the amplification and detection steps directly in a cell or tissue sample, including difficulties in controlling reaction conditions within the native tissue, as well as interference from other cellular

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components. Ensuring accurate replication of DNA, proper incorporation of chain-terminating nucleotides, and precise detection of fluorescent signals within the dense cellular matrix is technically infeasible. Therefore, despite its successful and routine application *ex situ*, performing Sanger sequencing *in situ* was not a logical next step.

359. Instead, decades of research have yielded technological advances and new sequencing technologies that overcome these limitations. For example, SOLiD sequencing by ligation is an alternative sequencing technology invented by George Church and colleagues in 2005. In contrast to Sanger sequencing, this method leverages the sequential ligation of fluorescent probes, and is more amenable to *in situ* applications. This shows that even when a molecular technology is successfully designed to analyze extracted molecules, it often requires significant redesign and technological innovation (over the course of decades) to be adapted for *in situ* applications.

360. In his report, Dr. Metzker argues that the combination of *in situ* art with array-based techniques was straightforward: “Using sequential imaging to provide a temporal order of signal signature was simply a matter of applying an algorithm to *in situ* methods already in use.” Metzker Rpt. at, for example, ¶ 206. I disagree. The extension of *in situ* methods that previously utilized a single round of hybridization to utilize combinatorial decoding techniques from array-based contexts is not simply a matter of applying an algorithm. A person of ordinary skill in the art would know that array-based techniques were developed and demonstrated in ways that leverage the artificial and highly controlled environment of an array. One of ordinary skill in the art would be aware of all of the many complications involved in moving from an array-based *ex situ* context to an *in situ* context and would not be motivated to combine such techniques, would not know what modifications would be required to adapt an array-based technique for use *in situ*, would not know

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if such an adaptation was even possible, and would not reasonably expect such a combination to succeed.

361. As I have previously explained, there are fundamental differences between analyzing target molecules in their native biological context (*in situ*) and analyzing target molecules that have been previously extracted from a sample (*ex situ*). Both Göransson (dissertation and Göransson 2009) and Gunderson (2004) present techniques for decoding a sequence of optical signatures produced from DNA molecules immobilized on a random array, not a cell or biological tissue sample. Moreover, both references discuss specific features and characteristics of array-based techniques that enable the success of their approaches.

362. For example, as discussed above the Göransson (dissertation) states: “Repetitive treatments of the arrayed ASMs put high demands on the array regarding attachment of the ASMs, as well as efficiency of the hybridization and dehybridization reactions.” Göransson Dissertation at 24, VIZ00026930-971 at 953 (emphasis added). A person of ordinary skill in the art would understand that the authors recognize that their combinatorial decoding techniques would not be successful if the ASMs were not attached and immobilized on the array, and additionally, if the hybridization and dehybridization reactions were not highly efficient. A person of ordinary skill in the art would recognize that an *ex situ* assay performed on an array presents an idealized environment, where users can immobilize target molecules, carefully control their density, optimize specific reaction conditions to maximize the efficiency of hybridization and dehybridization reactions, and minimize any background signal due to autofluorescence. A person of ordinary skill in the art would understand that each of these characteristics would not apply for an *in situ* assay where target molecules were present in a cell or biological tissue sample. Therefore, a person of ordinary skill in the art would understand that the requirements for the specific

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combinatorial decoding techniques presented in the array-based art could not be directly applied for *in situ* analysis.

363. Similarly, Gunderson (2004) teaches specific techniques that can ensure the reproducible generation of arrays where microbeads are fixed inside of etched wells with a pre-determined spacing and the microbeads are labeled with bead identification oligonucleotides. Arraying microbeads molecules in this highly controlled environment enables the decoding procedure, as the authors note: “High signal to noise allows the code of the circled bead to be read by eye”, and additionally, that “The clear separation between the two modes in the histogram indicates that most beads can be classified unambiguously as OFF or ON.” VIZ00026029-37 at 31. A person of ordinary skill in the art would understand that the high signal to noise ratio and unambiguous separation of fluorescent signals reported in Gunderson (2004) was reflective of the idealized and carefully controlled environment of the *ex situ* array.

364. A person of ordinary skill in the art would therefore understand that simply applying the techniques presented in Gunderson (2004) or Göransson (dissertation or Göransson 2009) to a cell or biological tissue sample, as Dr. Metzker suggests, would no longer benefit from the inherent advantages of the idealized array-based experimental conditions that are required for successful data generation. The non-uniform and uncontrolled density of target molecules, potential for spatial movement across hybridization cycles, specific challenges in maintaining signal intensity and quality across many hybridization rounds, and interference and background signal generated by additional molecules in the biological sample all represent substantial technical challenges that would preclude routine application of the array-based techniques for *in situ* experiments.

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365. Additional strong evidence that it was not obvious to modify or combine *in situ* techniques with sequential-detection techniques, including from array-based references, comes from Vizgen itself. Vizgen has, over the years, sought patent protection for MERFISH-related claims directed to the *in situ* use of sequential hybridization and detection techniques with priority dates *after* both the SeqTag Probe Patents and the prior art asserted by Vizgen in this litigation. For example, Vizgen applied for a patent during the litigation that was pending during the time Vizgen and Dr. Metzker have formulated invalidity theories against the SeqTag Probe patents. That patent, U.S. Patent No. 11,959,075, is directed to *in situ* detection of RNA using primary and secondary readout probes using sequential hybridization and imaging. U.S. Patent No. 11,959,075 (10XH-00358215-309). That patent claims priority to a provisional patent application with a September 15, 2014 date. *Id.* Independent claim 1 of the 075 Patent merely requires a plurality of codewords with Hamming weight 2, and Hamming distance 2 between those codewords. *Id.* Vizgen's founder, Dr. Zhuang, confirmed in deposition that this claim reflects an implementation of MERFISH. Zhuang Dep. 57:18-58:18.

366. [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

367.

368. Vizgen was confident in the validity of such claims because its founders and inventors did not believe it was obvious to combine Göransson with *in situ* prior art at all.

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369. Dr. Boettiger explained some reasons why it would not have been obvious to combine these techniques with any expectation of success—there were significant challenges and obstacles dissuading people from even trying to use Göransson’s *ex situ* methods in tissue. Boettiger Dep. 101:5-102:15.

370. Vizgen’s prosecution of its *in situ* sequential hybridization and imaging claims, and the Patent Office’s allowance of them over Göransson and Larsson, is significant evidence that such combinations were not obvious in 2012. If Dr. Metzker were correct about the scope of obviousness stemming from the asserted prior art, including Göransson and Larsson, Vizgen’s 075 Patent claims would be invalid as well. Vizgen’s strong belief outside of court that not only would these references not be combined or render the 075 Patent claims obvious, that there was not even a concern about validity for a 2014-dated application, shows that Dr. Metzker is wrong. This evidence from Vizgen also supports my opinions regarding objective indicia of non-obviousness which I address in more detail in a later section of this Report.

371. [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED] One of Dr. Cai’s methods for sequential FISH, or “SeqFish,” was published in Nature Methods in 2014. 10X595-00014628 (Cai Exh. 4). Dr. Cai’s method involved multiple rounds of hybridization of fluorescent probes to RNA targets, removal of probes, and sequential imaging, where a temporal code of colors corresponded to the readout from a given transcript.²⁴ *Id.* Dr. Cai

²⁴ I note that Dr. Cai described his sequential decoding approach as akin to “sequencing transcripts in single cells with FISH.” 10X595-00014630; *see also* Cai Dep. 35:14-36:3 (“This is basically sequencing by FISH in single cells.”). Dr. Cai’s description that in 2013 and 2014 he described combinatorial FISH barcoding as “sequencing” is further evidence that those skilled in the art in

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[REDACTED]

2. Additional Support for My Opinions Regarding the Lack of Motivation to Combine and the Lack of Reasonable Expectation of Success for Such a Combination Based on the Larsson Dissertation and/or Göransson 2009

372. My opinions regarding lack of motivation to combine, lack of guidance about how to make such a combination, and lack of reasonable success that such a combination would succeed are further supported by the Larsson Dissertation and by Göransson 2009.

the same field as ReadCoor, viewed sequential hybridization and imaging to identify transcripts as “sequencing” *in situ*.