

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

BRUKER SPATIAL BIOLOGY, INC.,
Petitioner

v.

PRESIDENT AND FELLOWS OF HARVARD COLLEGE and 10X
GENOMICS,
Patent Owner

Case IPR2024-00526
U.S. Patent No. 11,542,554

**DECLARATION OF HARI SHROFF, PH.D. IN SUPPORT OF PATENT
OWNER RESPONSE**

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10x Genomics EX2024
Bruker v. Harvard
IPR2024-00526

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I, Hari Shroff, hereby declare as follows.

I. INTRODUCTION

1. I have been retained as an expert witness on behalf of 10x Genomics, Inc. for the above-captioned *inter partes* review (IPR). I am being compensated for my time in connection with this IPR. I understand that this Declaration is being submitted in an *inter partes* review (IPR) proceeding involving U.S. Patent No. 11,542,554 (“the 554 patent”) (EX1001), which resulted from U.S. Patent Application No. 17/395,534 (“the 534 application”), filed on August 6, 2021. I understand that the 534 application is a continuation of U.S. Patent Application No. 16/386,337, filed on April 17, 2019 (issued as U.S. Patent No. 11,115,220), which is a continuation of U.S. Patent Application No. 15/969,118 filed on May 2, 2018 (issued as U.S. Patent No. 10,266,888), which is a continuation of PCT Application No. PCT/US2016/060279 filed on November 3, 2016. I understand that the PCT/US2016/060279 application claims benefit to U.S. Provisional Application No. 62/250,182 filed on November 3, 2015.

2. In preparing this Declaration, I have reviewed the 554 patent and each of the documents cited herein, in light of general knowledge in the art before November 3, 2015. In formulating my opinions, I have relied upon my experience, education, and knowledge in the relevant art. In formulating my opinions, I have also considered the viewpoint of a person of ordinary skill in the art (“POSA”)

prior to November 3, 2015.

II. MY BACKGROUND AND QUALIFICATIONS

3. I am currently a Senior Group Leader at the Janelia Research Campus of the Howard Hughes Medical Institute in Ashburn, VA. I also currently hold an appointment as an Adjunct Professor in the Department of Physics at the University of Maryland in College Park, MD.

4. Before becoming a Group Leader at Janelia, I was a Senior Investigator at the National Institute of Biomedical Imaging and Bioengineering, part of the National Institutes of Health from 2016-2022, where I pioneered computational microscopy techniques for improved spatiotemporal resolution, developed computational methods for accelerating post-processing of microscopy datasets, and developed deep-learning methods for denoising and enhancing the resolution of microscopic imaging. I also served as Director of the Advanced Imaging and Microscopy Facility at the National Institutes of Health from 2015-2022, where I helped to found a facility for translating home-built optical technologies developed in intramural labs for use by the broader NIH community. Furthermore, I served as Chief of the Section on High Resolution Optical Imaging at the National Institute of Biomedical Imaging and Bioengineering, a component of the National Institutes of Health from 2009-2022. Here I pioneered several microscopy systems including an improved 3D photoactivated localization

microscopy (PALM) system for super-resolution imaging in thick samples, a resolution-doubling structured illumination microscopy (SIM) system capable of imaging samples thicker than those imageable by then-existing commercial microscopes, and built a selective plane illumination microscopy system (SPIM) with improved axial and lateral resolution compared with previous methods.

5. Prior to my time as a Senior Investigator, I was a postdoctoral fellow at the Janelia Research Campus from 2006-2009. During my postdoctoral fellowship I performed the first dual-label PALM experiments on adhesion complexes and performed the first PALM experiments on living cells. This work enabled us to use fluorescence imaging to analyze the spatial distribution of adhesion complexes at the interface between cells and their substrata. During this time I also worked to improve and develop the PALM technology to make it broadly applicable to cell biology. Before this, I received my Ph.D. in Biophysics at the University of California at Berkeley in 2006. As part of my dissertation work, I developed a single molecule sensor of mechanical force based on fluorescent readouts. Prior to my graduate studies I began my undergraduate education at the University of Washington at the age of 14, graduating in 2001 with a B.S.E. in Bioengineering.

6. Over the years I have leveraged my experience with microscopy and fluorescence imaging to instruct graduate students, postdoctoral students,

undergraduate students, and faculty on modern imaging technologies and methods. For example, from 2013-2017, I instructed students on optics and modern fluorescence microscopy as Course Director of the annual Optical Microscopy in the Biomedical Sciences course in Woods Hole, MA. I was also an instructor for the Bangalore Microscopy Course at the National Center for Biological Sciences in Bangalore, India in 2014 and 2023. I have also taught courses on quantitative imaging at the Cold Spring Harbor Laboratory, Cold Spring Harbor, New York in both 2014 and 2019.

7. My work has been published in more than 100 peer-reviewed scientific articles that have been collectively cited more than 12,000 times. Since the beginning of my career I have presented more than 150 invited lectures or conference presentations on microscopy, imaging technologies, and my own research within related fields. These presentations include a recent presentation on New (& Old) Methods for Improving Fluorescence Imaging at Depth at the Computational Optics conference at the Janelia Research Campus in Ashburn, VA, a seminar on Multiscale Biological Imaging at High Spatiotemporal Resolution at the University of California, Los Angeles, and a keynote presentation on Live Cell Imaging Powered by Computation at the NHLBI Symposium on AI hosted by the National Institutes of Health at Bethesda, MA. My work has been the subject of several patents and patent applications on which I am listed as an inventor. These

patents include U.S. Patent No. 11,680,903, titled “Systems and methods for three-dimensional fluorescence polarization via multiview imaging,” and U.S. Patent No. 11,041,807, titled “Systems and methods for multi-view nonlinear optical imaging for improved signal-to-noise ratio and resolution in point scanning multi-photon microscopy.”

8. Throughout my career, I have received many awards and honors for my work, including the “Royal Microscopy Society Scientific Achievement Award” from the Royal Microscopy Society in 2022 and the National Heart, Lung, and Blood Institute’s Core Innovation Award in 2017 for work in the application of super-resolution imaging. In 2019 I was granted the Microscopy Society of America’s Burton Medal for distinguished contributions to microscopy by an individual less than 40 years of age. Furthermore, I was awarded the Anne Heidenthal Prize for Fluorescence Research in 2016.

9. My *curriculum vitae* is attached to this declaration as Exhibit 2025 and contains additional details on my education, honors and awards, publications, professional activities, and qualifications to deliver an expert opinion in this matter.

III. SUMMARY OF OPINIONS

10. In preparing this declaration, I have reviewed the declaration of Dr. Kirk Czymmek, Ph.D. (EX1002) and all the evidence cited therein. I have also

reviewed the references cited in my present Declaration. Dr. Czymmek asserts that the combination of Chen (EX1003), Shen (EX1032), and Cai (EX1004) would have rendered obvious the volumetric imaging system claimed in claim 36 of the 554 patent. EX1002, ¶¶124-219. As discussed herein, I disagree with Dr. Czymmek for at least the following reasons: (i) none of Dr. Czymmek’s references—alone or in combination—would have taught or suggested all of the elements of the claimed volumetric imaging system of claim 36 to a POSA (discussed in detail in Sections IX.B and IX.C), (ii) a POSA reading Chen and interested in using Chen’s highly multiplexed MERFISH setup would have had no reason to consult Cai or Shen (discussed in detail in Sections IX.A and IX.E.1-3), (iii) Dr. Czymmek does not explain what purported modifications a POSA would have made to Chen’s home-built imaging setup (discussed in detail in Sections IX.A and IX.E.4), and (iv) a POSA would have had no reasonable expectation of successfully modifying Chen’s MERFISH setup to create the claimed volumetric imaging system due to difficulties with massive multiplexing required in MERFISH (discussed in detail in Sections IX.A and IX.E.5).

IV. LIST OF DOCUMENTS CONSIDERED

Exhibit No.	Description
1001	U.S. Patent No. 11,542,554 (“the ’554 Patent”)
1002	Declaration of Dr. Kirk Czymmek, Ph.D. under 37 C.F.R. § 1.68

Exhibit No.	Description
1003	Kok Hao Chen et al., <i>Spatially resolved, highly multiplexed RNA profiling in single cells</i> , 348(6233) Science 412 (Research Article Summary) – aaa6090-1 - aaa6090-14 (2015) (“Zhuang”)
1004	U.S. Patent No. 10,457,980 to Long Cai et al. (“Cai”)
1006	Bioptechs, <i>FCS2 (Focht Live-Cell Chamber System) Instructions</i> , 1-6 (2007) at http://www.bioptechs.com/Instructions/FCS2_i/fcs2-3_i.htm (“FCS2 Instructions”)
1007	Sivaraj Sivaramakrishnan et al., <i>Shear Stress Induced Reorganization of the Keratin Intermediate Filament Network Requires Phosphorylation by Protein Kinase C ζ</i> , 20 Molecular Biology of the Cell 2755-2765 (2009) (“Sivaramakrishnan”)
1008	Chatarina Larsson et al., <i>In situ detection and genotyping of individual mRNA molecules</i> , 7 Nature Methods 395-397 (2010) (“Larsson”)
1010	Andrea M. Femino et al., “[13] Visualization of Single Molecules of mRNA in Situ,” 361 <i>Methods in Enzymology</i> , Elsevier Science (USA) 245-304 (2003) (“Femino”)
1011	Evan P. Perillo et al., <i>Enhanced 3D localization of individual RNA transcripts via astigmatic imaging</i> , 8950 SPIE 895003-1-895003-11 (2014) downloaded from https://www.researchgate.net/publication/263033754_Enhanced_3D_1ocalization_of_individual_RNA_transcripts_via_astigmatic_imaging (“Perillo”)
1012	Alison J. North, <i>Seeing is believing? A beginners’ guide to practical pitfalls in image acquisition</i> , 172 The Journal of Cell Biology 9-18 (2006) (“North”)
1018	Derek Toomre and Joerg Bewersdorf, <i>A New Wave of Cellular Imaging</i> , 26 Annu. Rev. Cell Dev. Biol. 285-287 (2010) (“Toomre”)

Exhibit No.	Description
1019	Robert S. Fischer et al., <i>Microscopy in 3D: a biologist's toolbox</i> , 21(12) Trends Cell Biol. 682-691 (2011) at https://pubmed.ncbi.nlm.nih.gov/22047760/ (“Fischer”)
1020	Sheel Shah et al., <i>Dynamics and spatial genomics of the nascent transcriptome by intron seqFISH</i> , 174(2) Cell 363-376 (2018) at https://pubmed.ncbi.nlm.nih.gov/29887381/ (“Shah”)
1021	Jeffrey R. Moffitt and Xiaowei Zhuang, <i>RNA Imaging with Multiplexed Error Robust Fluorescence in situ Hybridization</i> , 572 Methods Enzymol 1-49 (2016) at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5023431/ (“Moffitt”)
1027	Jie Xiao, “Single-Molecule Imaging in Live Cells,” <i>Handbook of Single-Molecule Biophysics</i> , Springer 43-93 (2009) (“Xiao”)
1032	Feimo Shen et al., “[32] Digital Autofocus Methods for Automated Microscopy,” 414 <i>Methods in Enzymology</i> , Elsevier Inc. 620-632 (2006) (“Shen”)
1036	Notice of Allowance issued by the United States Patent and Trademark Office dated June 21, 2022, during prosecution of U.S. Patent Application No. 17/395,534
1039	Kristen A. Brileya et al., <i>Bioflim growth mode promotes maximum carrying capacity and community stability during product inhibition syntrophy</i> , 5 Frontiers in Microbiology 1-13 (2014) (“Brileya”)
1041	U.S. Patent Application Publication No. US 2011/0216953 A1 to Daniel E. Callahan and Bahram Pravin (“Callahan”)
1042	U.S. Patent Application Publication No. US 2015/0098126 A1 to Phillip Johannes Keller and Raghav Kumar Chhetri (“Keller”)
1049	Leïla Tirichine et al., <i>3D fluorescent in situ hybridization using Arabidopsis leaf cryosections and isolated nuclei</i> , 5:11 Plant Methods 1-7 (2009) (“Tirichine”)

Exhibit No.	Description
1052	Patrick JM Zessin et al., <i>A hydrophilic gel matrix for single-molecule super-resolution microscopy</i> , 2:4 Optical Nanoscopy 1-8 (2013) (“Zessin”)
2006	Prosecution File History of U.S. Patent No. 11,542,554
2040	“Accelerating the Mastery of Biology to Advance Human Health,” 42 nd Annual J.P. Morgan Healthcare Conference, dated 2024 (accessible at https://s28.q4cdn.com/592666581/files/doc_presentations/2024/01/2024-TXG-JPM-Healthcare-Presentation-vF.pdf , last accessed December 9, 2024)
2041	Chen, K. H., “Spatially resolved, highly multiplexed RNA profiling in single cells,” Dissertation, Harvard University, April 2015
2042	Uchida, A., et al., “Live-cell imaging of neurofilament transport in cultured neurons,” <i>Methods Cell Biol.</i> , 131:21-90 (2016)
2044	Zhang, B., “Gaussian approximations of fluorescence microscope point-spread function models,” <i>Applied Optics</i> , 46(10):1819-1829 (April 1, 2007)
2045	Waters, J., “Accuracy and precision in quantitative fluorescence microscopy,” <i>The Journal of Cell Biology</i> 185(7):1135-1148 (2009)
2046	Conchello, J., et al., “Optical sectioning microscopy,” <i>Nature Methods</i> , 2(12):920-931
2047	Stallinga, S., et al., “Accuracy of the Gaussian Point Spread Function model in 2D localization microscopy,” <i>Optics Express</i> , 18(24):24461-22476 (November 22, 2010)

Exhibit No.	Description
2048	McCall, B., “FLUORESCENCE MICROSCOPY/QUANTITATIVE IMAGING: A systems-engineering approach to quantitative fluorescence bioimaging,” laserfocusworld.com, January 21, 2015 (accessible at https://www.laserfocusworld.com/biooptics/bioimaging/fluorescence/article/14190740/fluorescence-microscopy-quantitative-imaging-a-systems-engineering-approach-to-quantitative-fluorescence-bioimaging , last accessed December 9, 2024)
2049	Davis, A., et al., “Characterizing and Diminishing Autofluorescence in Formalin-fixed Paraffin-embedded Human Respiratory Tissue,” <i>Journal of Histochemistry & Cytochemistry</i> , 62(6):405-423 (2014)
2051	Lubeck, E., et al., “Single cell systems biology by super-resolution imaging and combinatorial labeling,” <i>Nat Methods</i> , 9(7):743-748 (June 2012), with supplemental materials
2052	Lubeck, E., et al., “Single-cell in situ RNA profiling by sequential hybridization,” <i>Nature Methods</i> , 11(4):360-361 (April 2014)
2053	Moffitt, J., et al., “High-throughput single-cell gene-expression profiling with multiplexed error-robust fluorescence in situ hybridization,” <i>PNAS</i> , 113(39):11046-11051 (September 27, 2016)
2054	Response to Office Action, U.S. Patent Application No. 17/374,000, filed January 26, 2024
2055	Response to Office Action, U.S. Patent Application No. 18/184,967, filed November 22, 2023
2057	U.S. Patent Application Publication No. 2012/0040397 A1 to Luo et al., published February 16, 2012
2058	U.S. Patent Application Publication No. 2014/0220587 A1 to Green, Jr. et al., published August 7, 2014

Exhibit No.	Description
2059	Chung, K., et al., “CLARITY for mapping the nervous system,” <i>Nature Methods</i> , 10(6):508-513 (2013)
2060	Xenium Analyzer User Guide, CG000584, Rev. G., 10x Genomics, Inc.
2061	CosMx® SMI Manual, MAN-10161-08, Software v1.4.1, Bruker Spatial Biology, Inc.
2062	Manual Slide Preparation, CosMX™ Spatial Molecular Imager, MAN-10159-01, NanoString Technologies, Inc.
2064	Cassini HW.V1 Design Review and Transfer to NPI, June 1, 2022, 10XH-00054907-96 (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2065	CosMx™ Spatial Molecular Imager Grant Support Document, NanoString Technologies, Inc.
2070	“New Insights Into Cleft Palate Unlocked With 10x Genomics’ Single Cell and Spatial Technologies,” 10x Genomics, Inc. Press Release, June 27, 2024
2071	“10x Genomics Surpasses 100 Xenium Analyzer Shipments,” BioSpace, August 29, 2023 (accessible at https://www.biospace.com/10x-genomics-surpasses-100-xenium-analyzer-shipments , last accessed November 24, 2024)
2072	Peroni, E., “Spatial-transcriptomic profiling: a new lens for understanding myelofibrosis pathophysiology,” <i>Cell Communication and Signalling</i> , 22:510 (2024)
2073	Cassini Phase 2 Design Review – Instrument and Software, July 13, 2022, 10X261-00003121-232 (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2074	Small, A., et al., “Fluorophore localization algorithms for super-resolution microscopy,” <i>Nature Methods</i> , 11(3):267-279 (March 2014)

Exhibit No.	Description
2075	Huang, F., et al., “Simultaneous multiple-emitter fitting for single molecule super-resolution imaging,” <i>Biomedical Optics Express</i> , 2(5):1377-1393 (May 1, 2011)
2076	Dobrucki, J., et al., “Scattering of Exciting Light by Live Cells in Fluorescence Confocal Imaging: Phototoxic Effects and Relevance for FRAP Studies,” <i>Biophysical Journal</i> , 93:1778-1786 (September 2007)
2077	Booth, M., “Adaptive optical microscopy: the ongoing quest for a perfect image,” <i>Light: Science & Applications</i> , 3, e165 (2014)
2078	Diaspro, A., et al., “Optical nanoscopy,” <i>La Rivista del Nuovo Cimento</i> , 43:385-455 (2020)
2079	Lee, J., et al., “Fluorescent <i>in situ</i> sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues,” <i>Nature Protocols</i> , 10(3):442-458 (2015)
2080	Schoder, D., et al., “Novel Approach for Assessing Performance of PCR Cyclers Used for Diagnostic Testing,” <i>Journal of Clinical Microbiology</i> , 43(6):2724-2728 (June 2005)
2081	Ettinger, A., et al., “Fluorescence Live Cell Imaging,” <i>Methods Cell Biol.</i> , 123:77-94 (2014)

V. LEGAL BASIS FOR MY ANALYSIS

A. Person of ordinary skill in the art

11. I understand that patent law analyses are performed from the viewpoint of a POSA. I understand that a POSA is a hypothetical person who is presumed to be aware of all pertinent art, who thinks along conventional wisdom in the art, and is a person of ordinary creativity. A POSA for the 554 patent would

typically have had a Ph.D. in molecular, cellular, or developmental biology and/or biophysics, bioengineering, biochemistry, or a related discipline, with at least 2 years of experience in the field of fluorescence imaging of biological materials, including cells and tissues. A POSA would have understood fluorescence imaging technologies and the use of microscopes to collect and record images. Furthermore, a POSA would have had some familiarity with gene amplification and sequencing technologies. I have reviewed the definition of a POSA in Dr. Czymmek's declaration. EX1002, ¶¶68-70. Dr. Czymmek's definition of a POSA is similar to my own, and I would have reached the same conclusions in my declaration had I applied Dr. Czymmek's definition.

B. Obviousness

12. I understand that a claim is unpatentable as obvious if the differences between the claimed invention and the asserted prior art are such that the subject matter as a whole would have been obvious to a POSA at the time the invention was made. Similarly, a claim is invalid as obvious if two or more prior-art references in combination disclose, expressly or inherently, every claim limitation so as to render the claim, as a whole, obvious to a POSA at the time the invention was made.

13. I understand that obviousness is ultimately a question of law based on a determination of a number of factual issues. Those factual issues relate to: (1) the

scope and content of the prior art, (2) the differences between the prior art and the claimed invention as a whole, (3) the level of ordinary skill in the art at the time the invention was made, and (4) secondary considerations that reflect, for example, the contemporaneous response to the inventions at the time, such as commercial success, long-felt need, failure of others, industry-wide praise, and unexpected results.

14. I understand that care must be taken to avoid the impermissible use of hindsight in an obviousness analysis. I understand that it is improper to use the invention as a plan or template for hindsight reconstruction of bits and pieces of the prior art to form the invention.

15. It is my further understanding that a patent claim composed of several elements is not proved obvious merely by demonstrating that each of its elements was independently known in the prior art. There must be a reason why it would have been obvious to modify the prior art to arrive at the claimed invention. I understand that the patent challenger has the burden to show that a POSA in the relevant field had a reason to combine the elements in the manner claimed when asserting obviousness in view of a combination of references.

16. I understand that there is no suggestion or motivation to make a modification to a prior-art reference if the proposed modification would render the prior-art invention unsatisfactory for its intended purpose. I also understand that an

obviousness allegation cannot be supported by a combination of references that would require a substantial reconstruction and redesign of the elements shown in the primary reference or a change in the basic principle under which the primary reference was designed to operate.

17. I understand that where there is a reason to modify or combine the prior art to achieve the claimed invention, there must also be a reasonable expectation of success in doing so. I also understand that conclusory statements lacking factual substantiation are insufficient for evaluating reasonable expectation of success as part of an obviousness determination. Instead, I understand that the Petitioner must articulate a basis upon which it concludes that it would have been obvious to make the claimed invention.

18. I have been informed that objective factors concerning non-obviousness, also known as “objective indicia,” may be probative of a claim’s obviousness. I have been informed that objective indicia can include such factors as contemporaneous responses to the inventions, commercial success, long-felt need, failure of others, industry-wide praise, and unexpected results. I have also been informed that, in order for the objective indicia to have weight, there must be a nexus between the evidence and the claim as a whole. I understand that, without a nexus, evidence of objective indicia does not support a finding of non-obviousness. I further understand that evidence of objective indicia will have no

weight if the evidence is related to an unclaimed feature or features found in the prior art.

VI. STATE OF THE ART

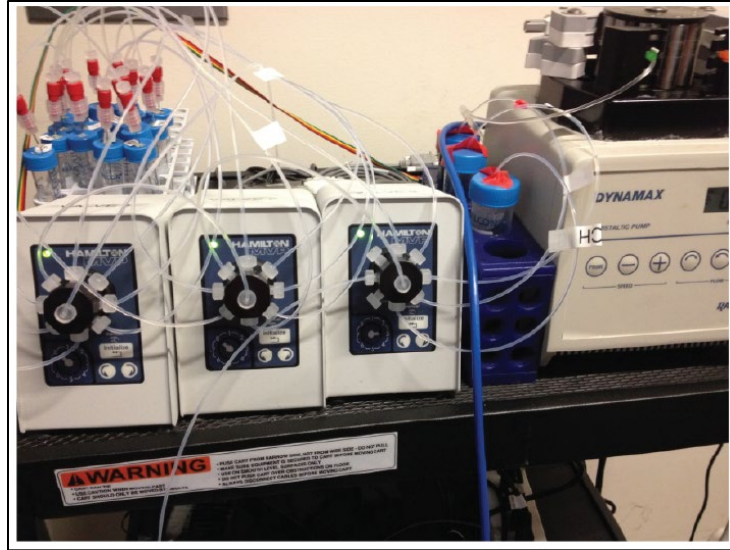
19. Before November 3, 2015, a POSA would have understood fundamental aspects of fluorescence microscopy that would have been instrumental for applications of single-molecule nucleic acid imaging. Dr. Czymmek relies on the prior art combination of Chen, Cai, and Shen to suggest that a POSA would have been motivated to modify Chen's 2D MERFISH imaging setup to create a system for volumetric imaging of individual nucleic acid molecules. In my opinion, however, fundamental aspects of single-molecule fluorescence microscopy—including axial resolution, the point-spread function, the signal-to-noise ratio, and refractive indices—would have been instrumental to a POSA's interpretations of the art and to any rationale for modifying Chen's 2D MERFISH imaging setup in view of the separate systems detailed in Cai and Shen. I will discuss each of these fundamental aspects and their relevance in turn below. This discussion will demonstrate why a POSA would not have modified Chen's 2D MERFISH imaging setup in view of the separate systems detailed in Cai and Shen.

A. Prior art systems for single-molecule imaging were unwieldy, home-built arrangements

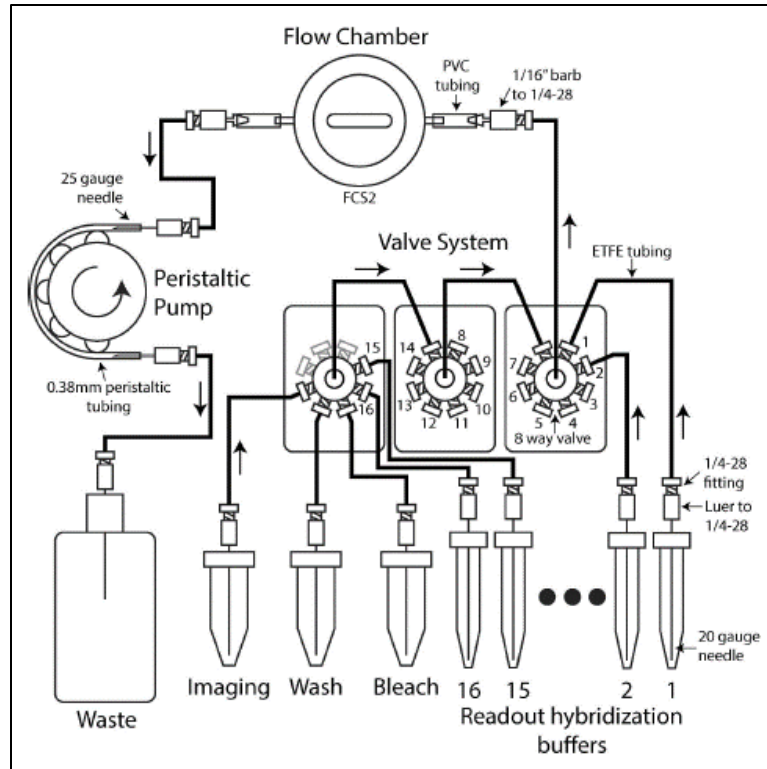
20. Before November 3, 2015, a POSA would have understood that

imaging systems in the prior art often varied in design based upon the intended use of the system. Furthermore, a POSA would have known that such imaging systems were frequently home-built setups that were unwieldy and not space efficient. The MERFISH setup constructed in Chen is an example of one such arrangement.

According to Chen, the arrangement was a “highly multiplexed smFISH imaging method that substantially increases the number of RNA species that can be simultaneously imaged in single cells.” EX1003, 002. This imaging arrangement comprised a “home-built” wide-field microscope for 2D imaging, a “home-built” autofocus system, and a “home-built fluidics system” comprised of three valves, a pump, and numerous tubes and bottles for the multi-round hybridization buffers and washes needed for 2D imaging of the MERFISH protocols. EX1003, 011; *see also* EX1021, FIG. 4. As I discuss in Sections IX.B and IX.D.3 below, Chen’s microscopy setup was designed for 2D imaging of fixed cells. *See infra*, Section IX.D.3. The first author of Chen was a graduate student in Xiaowei Zhuang’s lab who included many of the same disclosures from Chen in his dissertation. He also included a picture in his dissertation of the research group’s home-built MERFISH fluidics setup that depicts the numerous tubes, hoses, bottles, and pumps required for the protocol:



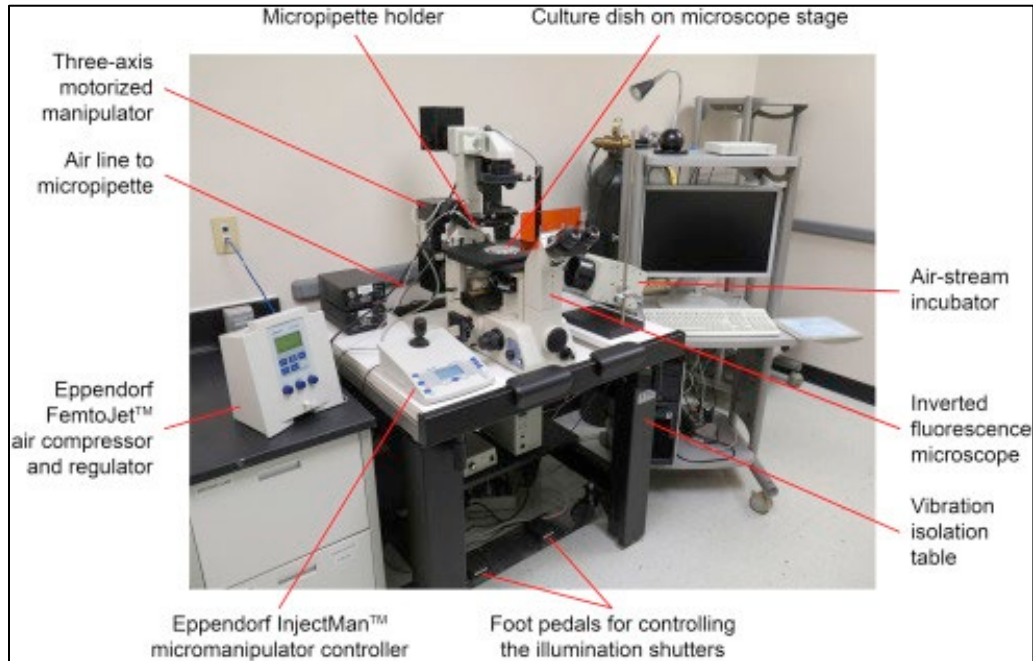
EX2041, FIG. 33. The image in the Chen dissertation shows only the fluidics portion of the setup. EX2041, FIG. 33. Moffitt later published a schematic of the same home-built setup depicted in Chen's dissertation. Moffitt's schematic, as shown below, depicts the setup with 16 tubes for different hybridization buffers ("Readout hybridization buffers"), three tubes for imaging, wash, and photobleaching buffers, and the tubing, valves, and the pump required for the fluidics system ("Peristaltic pump").



EX1021, FIG. 4. Such home-built imaging setups in the prior art were considered unwieldy, laborious to construct, and not suitable for all laboratory spaces. Such setups tend to be ad hoc arrangements that are neither easy to assemble nor easy to maintain. These difficulties render such home-built systems to be generally unsuitable for mass production or for adaptation as commercial products.

21. Other setups designed for 2D imaging were home-built as well. One such setup is that disclosed by Sivaramakrishnan, which uses a system comprising an “FCS2” flowcell that “maintains the cells and media at 37°C.” EX1007, 004, FIG. 8; *see also* EX1027, 098 (describing FCS2 as a “cell growth chamber”). Sivaramakrishnan further disclosed that the FCS2 chamber “was mounted onto the stage of an LSM 510Meta confocal microscope” and further fitted with “an air

stream stage incubator.” EX1007, 004. An example of a home-built imaging setup comprising an “air stream stage incubator” is depicted below:



See, e.g., EX2042, FIG. 6. As explained by Uchida, “[b]ecause the fan generates vibration, it must be isolated mechanically from the microscope,” so the authors “place it on an equipment shelf supported above the vibration isolation table top.” EX2042, 32. This vibration introduced by an external piece of hardware—the air stream stage incubator—demonstrates how home-built setups introduce additional assembly and maintenance challenges that render reproduction outside of the home lab challenging. Furthermore, these types of home-built arrangements often spanned entire benchtop spaces. This sprawling nature—combined with the difficulty of assembly and maintenance—meant that the setups were generally only useful for those researchers with access to the laboratory in which they were

housed.

B. POSAs would have understood that axial resolution is more difficult to achieve than lateral resolution

22. Before November 3, 2015, a POSA would have understood that resolution describes the minimum distance at which two objects can be distinguished from one another when imaged with a microscope. EX1018, 287. The resolution for any given microscope is limited by diffractive properties in a given medium, which cause otherwise point-like objects in a microscopic image to appear blurry rather than crisp. EX1018, 004. A POSA would also have understood the point-spread function (“PSF”) and its relationship to resolution in fluorescence microscopy. Resolution can be quantified through the PSF, a measure of the three-dimensional diffraction pattern of a point light source that effectively describes how blurry a point source appears when viewed under a microscope. EX1018, 004; EX1019, 009; EX1027, 023.

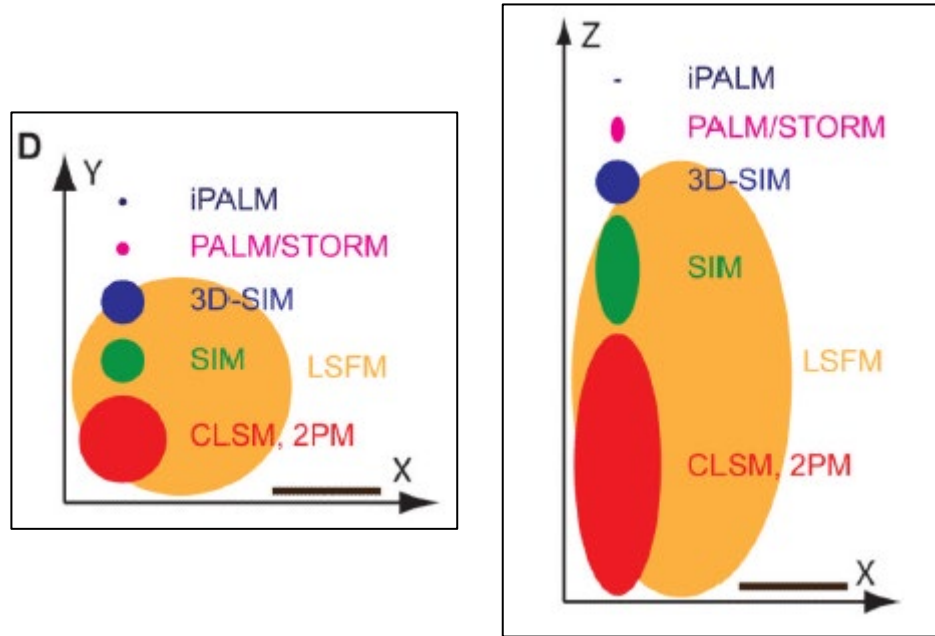
23. Before November, 3, 2015, a POSA would have understood that the PSF played a central role in understanding imaging performance of fluorescence microscopes. EX2044, 1819. For example, Zhang explains that “[t]he point-spread functions (PSFs) of fluorescence microscopes ... play a central role in understanding imaging performances, such as the theoretical resolution limit and the optical sectioning capacity” of the microscopes. EX2044, 1819. This central role of the PSF resulted in “a great amount of research ... focused on deriving

more and more accurate PSF models.” EX2044, 1819.

24. A POSA would have known that the PSF impacted axial resolution (z-axis) more substantially than lateral resolution (x-y axis). Namely, a POSA would have understood that the axial resolution of a conventional uni-axial microscope is typically around 2 to 3 times worse than the microscope’s lateral resolution. *See* EX1018, 004 (“axial resolution ... is typically 2 to 3 times worse than the lateral resolution”); *see also* EX2045, 1141 (“[r]esolution in the z-axis (z) is worse than lateral resolution in the light microscope.”). Furthermore, “the resolution along the Z-axis of the microscope is substantially ($\sim 2\times$) worse than in the lateral dimensions due to the inherent shape of the point spread function.” EX1019, 001. As a visual demonstration of this effect, panel D of the figure below shows the PSF of various microscopy methods in lateral dimensions (top right panel) and axial dimensions (bottom right panel). EX1019, 015. As shown in the figure, the effective PSF in the axial dimension is significantly elongated compared with the effective PSF in lateral dimensions. EX1019, FIG. 1D. Given this elongation in the axial range, resolving individual point sources of light along the z-axis was understood to be more challenging and complex than resolving individual point sources of light in the lateral (x-y) range, as the elongated PSF along the z-axis contributes to greater overlap with the diffraction patterns from other point sources along the z-axis and a concomitant increased difficulty in resolving individual point sources along that

axis. *See* EX1019, 001 (discussing the “Z-stretch” in fluorescence microscopy).

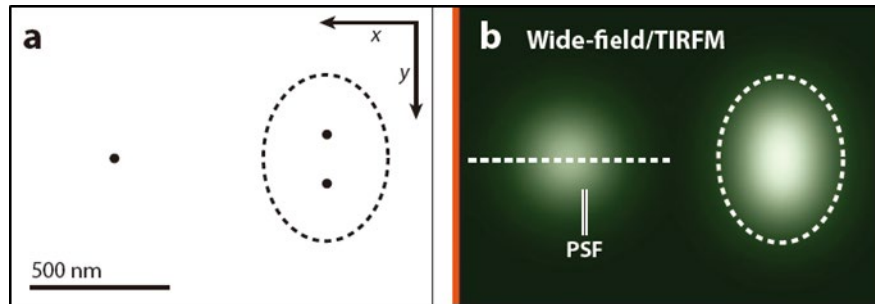
These trends in PSF for lateral and axial resolution would have been known to a POSA before November 3, 2015, and would have influenced the POSA’s understanding of the art.



EX1019, FIG. 1D.

25. The PSF determines the ability to resolve two point sources that are in close proximity to one another—if the distance between the two points is too small and the PSF is too large, then the blur generated from each point will overlap and the two points will not be resolvable. *See, e.g.,* EX1018, FIGs. 1A, 1B. As an example, in the left panel of the figure below, the single point on the left would be resolvable when viewed under a microscope (as seen in the right panel). This single point could be localized by determining the center of the PSF, which

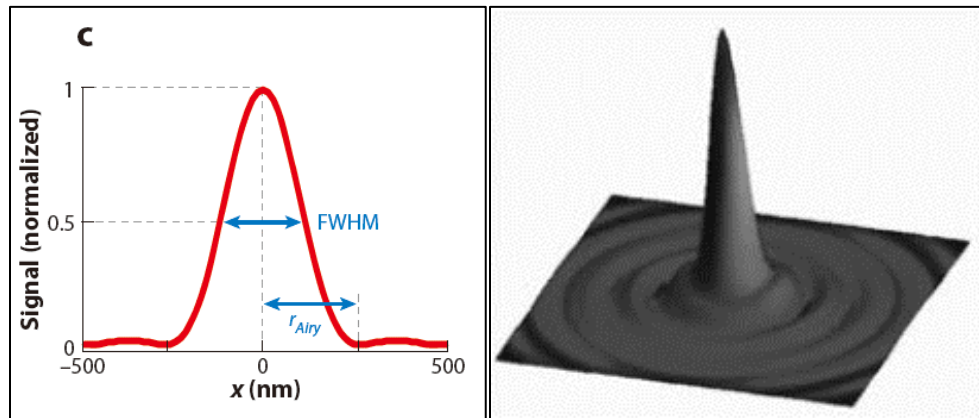
represents the localized position of the point source. EX1027, 023. In contrast, the two points on the right would not be resolvable, as the blur caused by the PSF for these closely-arranged points would overlap and cause the points to appear as a single blur under the microscope. EX1027, 023.



EX1018, FIG. 1A-B.

26. The PSF is a three-dimensional diffraction pattern of light from a point source in the specimen, as transmitted through a microscope's objective lens. EX1027, 003. The lateral cross-section of the PSF generates the shape of an Airy disk, as shown in Figure 1C and 1.2A below. The “full-width at half maximum” (FWHM) value of the Airy disk represents an estimate of the size of the PSF. EX1018, 004. The FWHM represents the spread at which the peak intensity falls to half of its value on each side of the Airy disk's peak, and a POSA would have understood that the FWHM was a standard measure used to characterize the resolution of a given fluorescence microscope. EX1018, 004. The FWHM value may be used to approximate whether two points are resolvable, with two points generally being resolvable if the distance between them is greater than or equal to

the FWHM of the PSF. EX1018, 003.



EX1018, FIG. 1C (left), FIG. 1.2A (right).

27. A POSA would have known that the PSF is influenced by several factors, including the numerical aperture (“NA”) of the microscope’s objective lens, the wavelength of emission light (e.g., fluorescence) from the sample, and the refractive index of the sample, the imaging medium, and the objective lens.

EX1027, 023; EX1018, 024. A POSA would have also known that the relationship between these factors is reflected in the equations for lateral resolution ($r = 0.61\lambda/NA$) and axial resolution ($z = n\lambda/NA^2$) and would have been able to understand how each factor influenced resolution in each dimension, as explained below. EX1018, 004.

28. A POSA would have understood that two points are resolvable if the distance d between them is greater than or equal to the radius of the Airy disk-shaped PSF. EX1018, 004. For lateral resolution, this radius is approximated by the formula $r = 0.61\lambda/NA$, wherein λ represents the wavelength of emission light

and NA is the numerical aperture of the objective lens. EX1018, 004. In contrast, axial resolution is approximated by the formula $z = n\lambda/NA^2$, wherein n is the refractive index of the specimen. EX1018, 004. Smaller values of r and z , respectively, increase the resolution of a microscope by decreasing the required minimum distance before two points become resolvable. *See* EX1018, 004. From these equations, a POSA would have understood that smaller wavelengths (λ) tighten the resolution by decreasing the numerator of the equations, while a higher NA increases resolution by increasing the denominator of the equations. *See* EX2046, 922. Taken together, a POSA would have known that the NA of the microscope objective lens, the wavelength of light used for imaging, and the refractive index of the sample and imaging medium are all relevant in determining the PSF—and therefore the resolution—of a microscope.

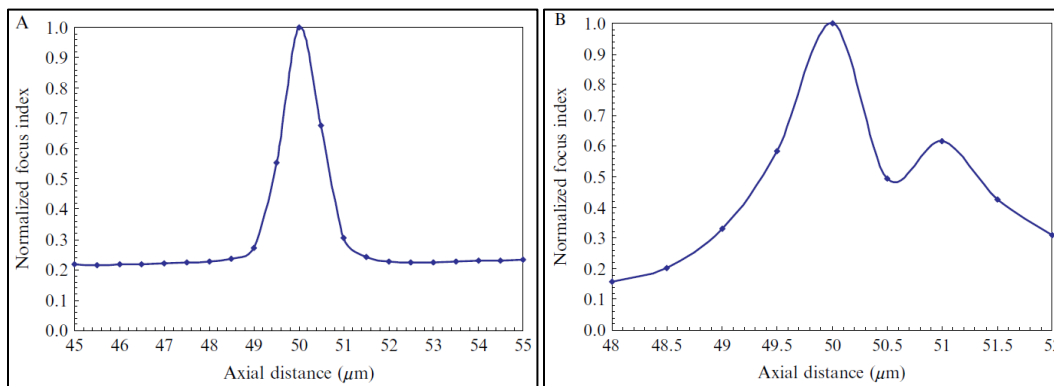
29. POSAs knew that approximating the PSF of a point source could be achieved using a “Gaussian distribution” or “Gaussian function.” EX2074, 268; EX2075, 1380. The 2D Gaussian function is a mathematical model that describes how a point source of light in a sample is spread out in an imaging plane. EX2047, Abstract. The Gaussian curve takes on the shape of a bell curve—similar to that of the Airy disk described above—and serves as an effective approximation of the PSF that is typically less computationally demanding than PSF calculations based on the Airy disk. EX2047, FIG. 1; EX2074, 268. To approximate a PSF value with

a Gaussian curve, the curve must be “fit” to the underlying data using one of several fitting criteria, including the maximum-likelihood criterion or the least-squares criterion. EX2074, 269.

30. A POSA would have understood that the Gaussian function was a commonly used model “for fitting the position of fluorescent emitters in localization microscopy.” EX2047, Abstract. Small explains, however, that while the Gaussian function “gives useful and reasonably accurate results in many investigations involving focused images of fluorophores,” the utility of the function can decrease in instances where the image is defocused, as often found in 3D imaging or experiments where thick samples are used. EX2074, 268; *See also* EX1032, 007. Small further explains that “almost any realistic PSF will look roughly similar to a Gaussian bell curve near the peak of a focused image ... which is the main reason why the Gaussian approximation gives useful and reasonably accurate results in many investigations involving focused images of fluorophores.” EX2074, 268. However, a POSA would also have understood that “[i]f, however, the image is defocused (a common situation in 3D), then the PSF shape can be considerably more complicated,” thereby decreasing the potential utility of the Gaussian function. EX2074, 268.

31. A POSA would have known that the Gaussian function is affected by sample thickness. For example, a POSA would have known that “[t]hin specimens

produce an ideal curve that can be fit readily to a Gaussian [function],” as demonstrated by the single strong peak—like that seen in a typical Airy disk—in the left panel of the figure below. EX1032, 007, FIG. 2. In contrast, a POSA would have known that “when thicker specimens have discernible objects in different planes, multiple peaks can be produced in the curve.” EX1032, 007. These multiple peaks, illustrated in the right panel of the figure below, create additional difficulty in fitting the curve to a Gaussian function. For example, a POSA would have known that difficulties are frequently encountered in images “taken using high NA lenses because the DOF [depth of field] is much less than the cell thickness.” EX1032, 007.



EX1032, FIG. 2. A POSA also would have known that fitting a 2D Gaussian curve was relatively straightforward in comparison to fitting a 3D Gaussian curve, as fitting a 3D function requires different—and more complex—computational methods to adjust for factors including the “Z-stretch” of the PSF and the associated reduced axial resolution. EX1019, 001; EX1018, 004; EX2045.

C. POSAs were aware of various other factors that were important for axial resolution, such as the signal-to-noise ratio, autofluorescence, background fluorescence, and signal density

32. In addition to the point-spread function and the related factors discussed above, a POSA would have known that other aspects of fluorescence microscopy would influence axial resolution in single-molecule imaging.

33. **Signal-to-Noise Ratio and Signal-to-Background Ratio.** A POSA would have had a strong understanding of the signal-to-noise ratio (“SNR”) and its impact on fluorescence microscopy. In fluorescence microscopy, background fluorescence “causes imprecision in measurements of pixel intensity values, and therefore a level of uncertainty in the accuracy of the measurements.” EX2045, 1136. A POSA would have known that to detect a signal’s presence, “the signal must be significantly higher than the noise level of the digital image.” EX2045, 1136. In general, a high SNR occurs when the signal is strong in comparison with the background noise in a sample, causing measurements to be more precise. EX2045, 1136. In contrast, a low SNR can occur when there is more background noise in the sample, making the signal more difficult to distinguish from background noise. EX2045, 1136. Similarly, a POSA would have known that high background—resulting in a low signal-to-background ratio (SBR)—can affect image quality and the ability to discern individual fluorescently labelled molecules. *See* EX2048, 3 (discussing how “many of the same factors that affect the amount

of fluorescence signal detected also affect the amount of background light detected.”). 6 (noting that “increasing both SBR and SNR is important for precisely and accurately detecting and quantifying the presence of biomolecules”). A POSA would have known that precise localization of a fluorescently labelled object—such as a nucleic acid—depends heavily on the SNR. EX2045, 1136. For example, a POSA would have understood that “SNR affects spatial measurements as well as intensity measurements; precise determination of the location of a fluorescently labelled object depends on SNR.” EX2045, 1136.

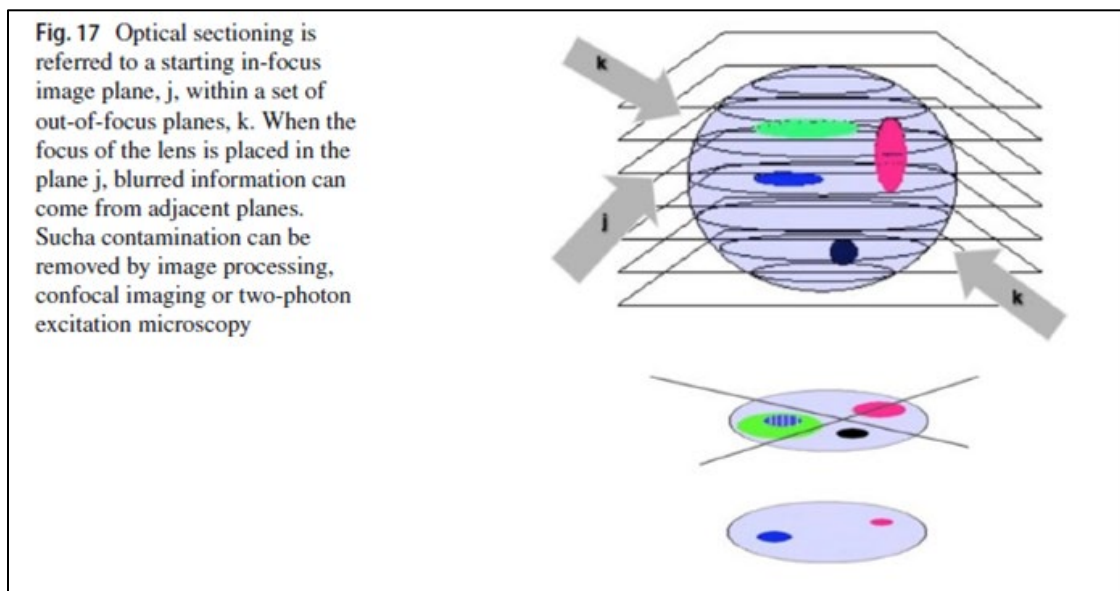
34. A POSA would also have understood the impact of background fluorescence on fluorescence microscopy. In fluorescence imaging, an illuminating light with an excitation wavelength capable of inducing a sample to fluoresce is focused at the image focal plane by the objective lens of the microscope. EX2045, 1139. This will maximally excite fluorophores at the plane where the excitation light is focused, or the image plane. EX2045, 1139. Although focusing light on the image plane selectively excites fluorophores on that plane, background noise frequently occurs as fluorophores in the sample both above and below the focal plane are excited by the illuminating light as well. EX2045, 1139. Light emitted by these out-of-focus fluorophores can then be collected by the microscope’s objective lens, where it will appear as out-of-focus background fluorescence in the image focal plane itself. EX2045, 1139. The background fluorescence caused by

out-of-focus fluorophores impairs the quality of the image obtained from fluorescence imaging by increasing the amount of noise in the sample, thereby decreasing the SNR. EX1019, 002. This added background from out-of-focus fluorescence occurs regardless of where a microscope is focused vertically within a specimen. EX2046, 920. The prevalence of background fluorescence from out-of-focus illumination and excitation is so pervasive that more than 90% of fluorescence in a given 2D image can come from out-of-focus light. EX2046, 920.

35. In addition to background coming directly to the objective lens from out-of-focus fluorophores, scattered fluorescence from a sample can also work to decrease SNR and contrast. EX2046, 920; *see also* EX2076, 1778 (discussing how “[i]n wide-field fluorescence microscopy ... scattered fluorescence is seen as a bright background” that “causes deterioration of image contrast and, thus, degrades image resolution.”). A POSA would have known that scattered light from background fluorescence could impact the SNR when fluorescent emissions from a sample are diffracted, reflected, and refracted throughout a sample and make their way to the microscope’s objective lens. EX2046, 920; *see also* EX2076, 1778. Light scattered through these mechanisms appears to have been emitted from the last point of scattering in the sample. EX2046, 920; *see also* EX2076, 1778. Any light whose last point of scattering was at or near the image focal plane adds to the background noise and negatively impacts the SNR. This effect is further

pronounced in thicker samples, where light has increased chances to scatter throughout the many focal layers of the sample. EX2046, 920; *see also* EX2076, 1778; *see also* EX2077, 4 (describing brain tissues as “thick, strongly scattering specimens”); EX2077, 6 (discussing that “[i]ncreasing the image depth within a specimen should [] lead to a greater scattering contribution”). A POSA would have been aware that such challenges would impact any attempts to perform fluorescence imaging, particularly in thick samples.

36. Optical sectioning is one technique for improving SNR and SBR that would have been understood by a POSA. EX2046, 920. Optical sectioning techniques acquire images of thin slices of an otherwise thick specimen while removing fluorescence contributions by out-of-focus light in the image plane. EX2046, 920. This basic process is depicted in the figure below:



EX2078, 25. As illustrated above, optical sectioning involves focusing a

microscope's objective on a single plane (plane j in the figure) while removing background fluorescence sourced from other planes in a thick sample (for example, out-of-focus planes k in the figure above). EX2078, 25. Background fluorescence from out-of-focus planes can be removed through several means, including through computational optical sectioning, confocal microscopy, and light sheet microscopy. Computational optical sectioning leverages digital image processing algorithms to remove background fluorescence virtually. EX2078, 23. In contrast, confocal microscopy physically excludes out-of-focus fluorescence using a pinhole aperture, with the size of the pinhole affecting both the rejection of background fluorescence as well as the quality of the image. EX2046, 926; *see also* EX2076, 1778 (discussing that in a confocal microscope "scattered fluorescence is eliminated from the image by a confocal aperture.").

37. **Autofluorescence.** A POSA would have known that autofluorescence is yet another common contributor to background noise and a lowered SNR in fluorescence imaging. EX1010, 022. Namely, a POSA would have known that autofluorescence is a major source of background that is inherent to biological samples. EX1010, 022. Autofluorescence is caused by biological components with fluorescence absorption or emission properties in the same frequency range as the fluorescent probes used in a given fluorescence imaging procedure. EX1010, 024; EX1012, 004. Sources of autofluorescence in human tissues include a wide variety

of tissue components, such as collagen, lipofuscin, elastin, and red blood cells.

EX2049, 001. Residual components of tissue processing techniques—such as formalin—as well as reagents such as serum can also contribute to issues with autofluorescence in tissue samples. EX2049, 001. Autofluorescence frequently occurs with proteins in animal tissues, as well as in cultures containing dead or dying cells. EX1012, 004. Autofluorescence from some sources can rival or exceed the intensity of well-designed fluorescent probes. EX1010, 026.

38. **Signal Density.** Signal density is another factor that a POSA would have understood to influence the resolution of a microscope. Signal density in microscopy is a measure of how many individual signals are detected and imaged within a single focal plane. If too many signals are located in close proximity to each other, then signal density is increased and it becomes difficult to discern individual signals. *See* EX2051, 3 (noting that “[w]hen the density of transcripts is high, diffraction-limited fluorescent spots of the same color will overlap and make barcode readout impossible.”). A POSA would have known that increased multiplexing—generating more signal by simultaneously tagging a greater number of nucleic acid species—can increase the signal density while making it harder to discern signal from individual molecules. This understanding was well established in the art, as demonstrated by Chen’s disclosure that “[a]s the degree of multiplexing is increased, it is important to consider the potential increase in the

density of RNAs that need to be resolved in each round of imaging.” EX1003, 008.

The consequences of this increased drive toward multiplexing on resolution are further discussed in Lubeck, where the authors recognize that “[w]hen the density of transcripts is high, diffraction-limited fluorescent spots of the same color will overlap and make barcode readout impossible.” EX2051, 003. This same concern is also recognized in Cai, where the authors note that “[b]ecause smFISH often uses two or more colors for a target, it produces high density of objects in the image” and this high density of objects “can increase the complexity of data analysis.” EX1004, 1:67-2:3. In particular, scaling up spatial barcoding techniques creates significant costs for researchers and can require more sophisticated algorithms to handle the computational load. *See, e.g.*, EX2051, 7 (discussing that scaling a spatial barcoding technique “beyond 1,000 genes” required a “more sophisticated computational algorithm”). POSAs of the time would have understood the potential negative impacts of increased multiplexing on the resolution of fluorescence imaging techniques such as smFISH. Furthermore, the POSA would have known that the aspects of fluorescence imaging discussed above—including the PSF, SNR, signal density, and autofluorescence—would have a particularly strong impact on axial resolution (z-axis) when compared with lateral resolution (x-y axis).

D. MERFISH was reported as improved multiplexing over other FISH methodologies

39. Fluorescence in-situ hybridization (FISH) techniques are a group of fluorescence microscopy techniques that would have been available to a POSA before November 3, 2015. FISH techniques involve labelling nucleic acids in a sample with fluorescent probes, which are subsequently imaged under a fluorescence microscope. While many varieties of FISH would be known to a POSA, relevant here are three varieties disclosed in the prior art combination relied upon by Dr. Czymmek: single molecule FISH (smFISH), sequential FISH (seqFISH), and multiplexed error robust FISH (MERFISH).

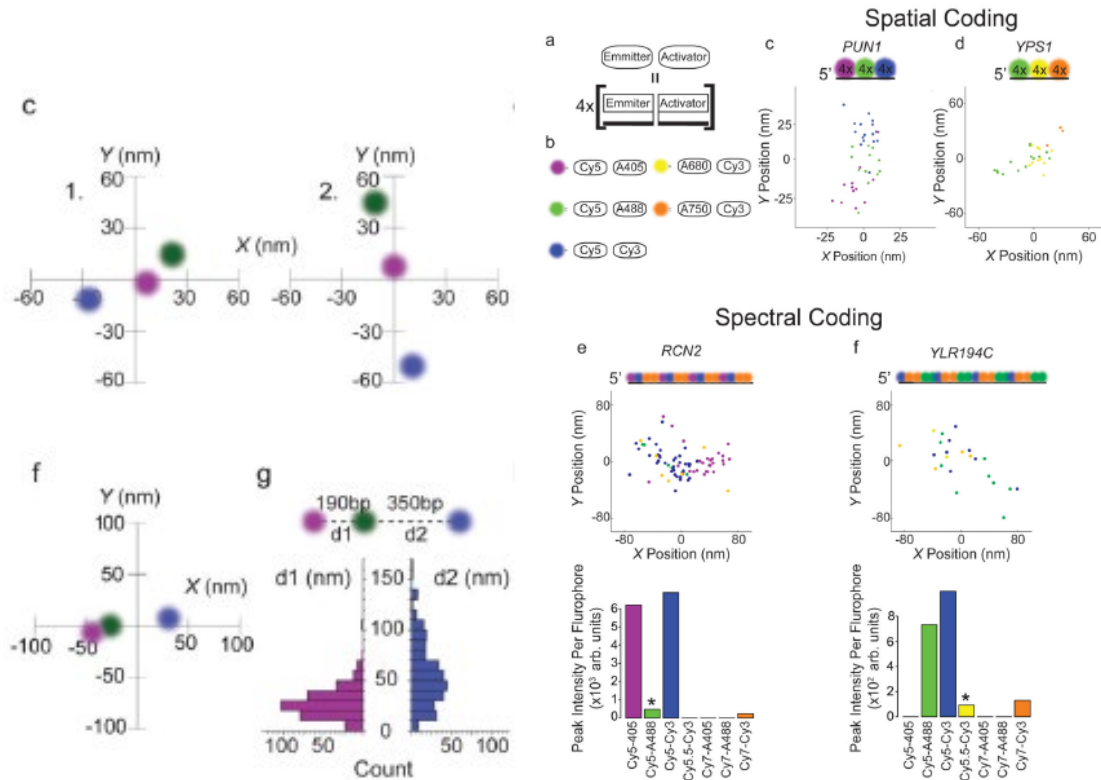
1. Single-molecule FISH

40. Lubeck disclosed methods for using FISH to simultaneously image multiple different mRNA transcripts in the context of yeast cells. EX2051. Lubeck's method involved targeting mRNA with multiple distinct sets of probes, each labeled with different fluorophores. EX2051, 2. The two-dimensional localization of targeted mRNA molecules was then resolved by spectral barcoding. EX2051, 4. Namely, the individual transcripts were coupled to a "barcode" combination of particular fluorophores, allowing the researchers to identify mRNA species by the combination of fluorescent signals located at any individual point in a sample. However, Lubeck also disclosed that "the number of molecular species that can be measured simultaneously is limited by the spectral separability of

fluorophores.” EX2051, Abstract. This combination of spectral barcodes allowed the author’s smFISH method to “increase the capacity for multiplex detection of molecules in single cells” by combining these spectral barcodes and super-resolution microscopy. EX2051, Abstract.

41. Lubeck reported measuring the mRNA levels of 32 genes simultaneously in single *S. cerevisiae* cells using sets of fluorophore pairs. EX2051, 6-7. Lubeck’s barcoding design required the use of probes that, when paired together, constituted a photoswitchable pair. EX2051, 3. With this setup, each probe in the pair would need to be hybridized next to each other on the target molecule before their associated fluorophore could be activated. EX2051, 3. Lubeck used the disclosed smFISH methodology to assess the localization of mRNA in two dimensions. EX2051, 3. A POSA would have understood that the two-dimensional nature of Lubeck’s protocol is shown by Lubeck paper’s discussion of the requirements for the protocol. For example, Lubeck acknowledged that “for two-dimensional imaging the spatial barcoding scheme necessitates the linearization of mRNA.” EX2051, 3.

42. Figures 1 and 2 in Lubeck, as shown below, further illustrate the two-dimensional nature of the results obtained from Lubeck’s smFISH procedures:



EX2051, FIGs. 1-2. Lubeck's Figure 1 (in particular panels 1C and 1F) shows the x and y coordinates of three fluorophores hybridized to mRNA and localized via a 2D Gaussian fitting. EX2051, FIGs. 1, 12. Figure 2 takes this further, showing x-y coordinate mapping for several mRNA spatial and spectral barcodes. EX2051, FIGs. 2C, 2E, 2F. Thus, Lubeck 2012's disclosures of smFISH were designed for 2D localization of mRNA molecules.

43. Lubeck's use of smFISH for 2D imaging is consistent with other sources in the art. For example, Perillo notes that "[t]wo drawbacks of the smFISH technique are 1) it is necessary to fix and permeabilize cells prior to labeling, requiring a number of independent experiments to obtain temporal dynamics and

2) the *data is primarily two dimensional*, due to the lack of axial resolution typical of wide-field imaging.”¹ EX1011, 002; *see also* EX1012, 004 (“wide-field images can provide clear lateral (x- and y- axis) information but only limited axial (z-axis) information.”); EX2079, 446 (characterizing wide-field epifluorescence microscopes as having “poor axial resolution”). Applying this general understanding, a POSA would have been aware that smFISH was a 2D imaging methodology with distinct challenges related to any proposed 3D implementation.

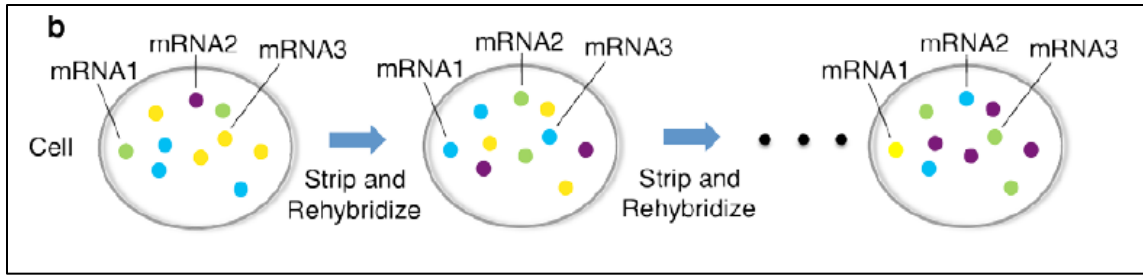
2. Sequential FISH

44. The Lubeck research group later built upon their work to create seqFISH, a “sequential barcoding scheme to multiplex different mRNAs.” EX2052, 360. This new seqFISH method involved fixing mRNA transcripts in cells and barcoding those mRNAs through “sequential rounds of hybridization, imaging and probe stripping.” EX2052, 360. By fixing the mRNA transcripts within the cells, “the corresponding fluorescent spots remain in place during multiple rounds of hybridization and can be aligned to read out a fluorophore sequence.” EX2052, 360. In effect, the sequential rounds of hybridization and imaging create a unique fluorescent “barcode” that uniquely identifies the species of mRNA targeted by the probes. The resulting fluorescent map of mRNA

¹ Emphasis is added throughout except where otherwise indicated.

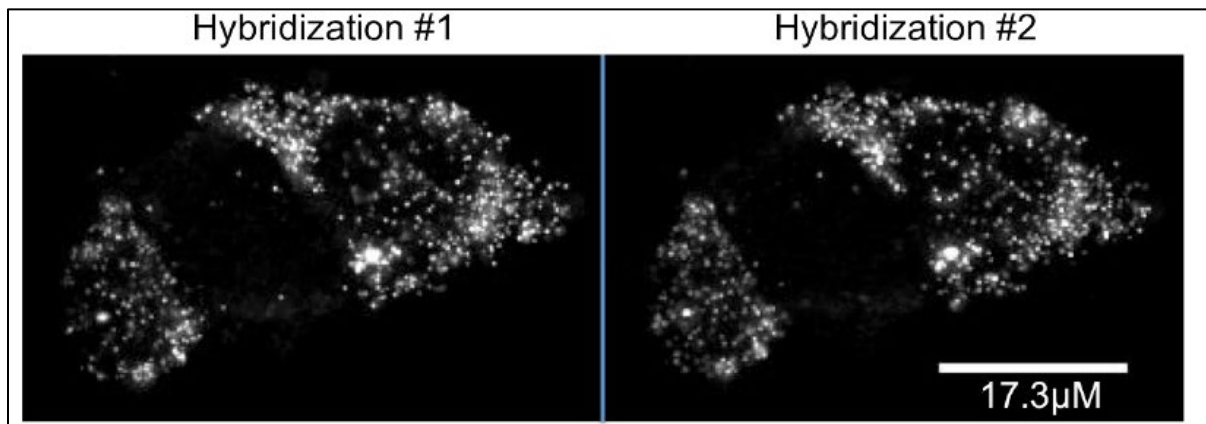
signals—as shown in Figure 1 of the paper—can then be used to localize targeted mRNA molecules in two dimensions. *See* EX2052, FIG. 1.

45. One of the major drawbacks of the seqFISH process that a POSA would have understood is the length and tediousness of the process. Performing seqFISH requires overnight hybridizations of probes followed by washing, imaging, and then a stripping procedure to remove the probes. EX2052, 14 (“Each round of hybridization took place overnight on the microscope, with DNase treatment and imaging occurring during the day.”); EX1004, 38:60-39:27. After this process was completed, the next overnight hybridization step would follow and the entire process would be repeated for the desired number of iterations, so that an experiment with 6 rounds of hybridization would take at least 6 days to perform. *See* EX2052, 14 (disclosing that “[u]p to six rounds of hybridizations were carried out on the same sample.”). As with their publication on smFISH, Lubeck 2014 disclosed using seqFISH to assess 2D localization of mRNAs in yeast and mouse cells. For example, Supplemental Figure 1B—a schematic of the FISH imaging procedure—shows localization of three mRNA species within a cell in two dimensions:



EX2052, FIG. S1B.

Likewise, Supplemental Figure 10 further demonstrates representative 2D maximum intensity projections following two different hybridization rounds, highlighting the 2D nature of most seqFISH imaging:



EX2052, FIG. S10; *see also* EX1004, FIG. 2B (composite four-color hybridization images).

46. Despite purported increases in multiplexing ability provided by the seqFISH method, a POSA would have recognized that seqFISH was still limited to imaging around 30 mRNA species in a given experiment. EX1003, 002. This understanding is demonstrated by Chen in 2015, where Chen notes that “efforts by using combinatorial labeling with either color-based barcodes or sequential

hybridization have enabled simultaneous measurements of 10 to 30 different RNA species in individual cells.” EX1003, 002 (citing EX2051 and EX2052). Moffitt later confirmed this in their 2016 publication when they disclosed the limited multiplexing capability of seqFISH, adding that “even the most advanced multiplexing efforts have only extended this approach to the simultaneous measurement of ~10–30 RNA species.” EX1021, 002 (citing EX2051 and EX2052). This limitation on multiplexing was in part due to the concomitant increase in detection error rates with increased numbers of distinct signals used in an experiment. EX1003, 002, FIG. 1D. In effect, while increasing the number of distinct signals in a seqFISH procedure increased the number of addressable RNA species, the detection error rates would also grow at the same time. EX1003, 002, FIG. 1D. A POSA would have understood these challenges and would have considered them when approaching the later MERFISH method detailed below.

3. Multiplexed Error-Robust FISH

47. In 2015, Chen et al. disclosed a method for “multiplexed error-robust FISH (MERFISH),” which was a “highly multiplexed smFISH imaging method that substantially increases the number of RNA species that can be simultaneously imaged in single cells.” EX1003, 002. This procedure achieved highly multiplexed imaging of mRNA by using a two-step labeling scheme with two different categories of probes, described in Chen as utilizing “combinatorial labeling and

sequential imaging with error-robust encoding schemes.” EX1003, 002. First, cellular RNAs were labelled with a set of encoding probes, with each probe containing two readout sequences and each RNA species being assigned four distinct readout sequences total. EX1003, 003. Next, multiple rounds of hybridization with readout probes targeting specific readout sequences would be performed and fluorescently imaged. EX1003, 003. This MERFISH method provided the ability to measure over 1,000 RNA species in a single experiment, and according to the authors had the capability of performing up to 16 rounds of hybridization, imaging, and photobleaching within approximately 20 hours. EX1003, 007-008, 012.

48. A POSA would have understood that Chen’s objective in MERFISH *was to increase the multiplexing capability of FISH techniques*. In other words, a POSA would have understood that *Chen wanted to increase the number of different mRNA species that could be detected simultaneously*. For example, Chen’s description of their technique as a “highly multiplexed smFISH imaging method that substantially *increases* the number of RNA species that can be simultaneously imaged in single cells” highlights the increase in the number of RNA species that can be imaged in cells—i.e. the multiplexity of the method. EX1003, 002. Chen further indicates that the researchers “sought to *further increase* the throughput of our MERFISH measurement by simultaneously imaging

~1000 RNA species” and that “it should be possible to *further increase* the number of detectable RNA species by using MERFISH with either MHD4 or MHD2 codes.” EX1003, 007-008. It is my opinion that a POSA would have recognized the above disclosures to show that Chen’s primary objective in developing MERFISH was increasing multiplexing for FISH techniques.

49. The objective of increasing multiplexity in MERFISH is further demonstrated by the research group’s later publication and patent applications directed to MERFISH. For example, in 2016, the group reported “an improved MERFISH method that drastically *increases the throughput*” of the procedure. EX2053, 11047. I understand that the same research group has also applied for several patents directed to MERFISH. I understand from reviewing portions of the file histories of U.S. Patent Application Nos. 17/374,000 and 18/184,967 that the research group acknowledged the limitations in multiplexity of the smFISH and seqFISH methods that MERFISH was intended to address. For example, in remarks on claim amendments for Application No. 17/374,000, the research group outlined that:

Chen demonstrates that **multiplexing requires an error robust coding scheme** to accurately image spatial organization of RNA transcripts *in situ* and that **the methods of seqFISH** (See Lubeck and Cai, *Nat. Methods*, 9(7):743-748, 2012 and, Lubeck and Cai, *Nat. Methods*,

11:360-361, 2014) **are inadequate for those purposes** (“scaling up the throughput of smFISH to the systems scale faces significant challenge because not only does the addressable RNA species increases [sic] exponentially with N [each RNA species by multiple (N) distinct signals], but the detection error rates also grow exponentially with N). *See* Chen at p. 2, “Combinatorial labeling with error-robust encoding schemes.” Moreover, “as N increases, the fraction of RNAs properly detected (the calling rate) would rapidly decrease and, more troubling, the fraction of RNAs that are identified as incorrect species (the misidentification rate) would rapidly increase,” wherein “the majority of RNA molecules would be misidentified after 16 rounds of hybridizations!”

To address that problem of using smFISH probes in multiplex assays, Chen developed error-robust encoding schemes, in which only a subset of 2^N-1 words separated by a certain Hamming distance are used to encode RNAs. This was exemplified with a coding scheme “in which the number of 1 bits [Hamming weight] were kept both constant and relatively low-only four per word in this work-so as to reduce error and avoid biased detection.” *See* Chen at p. 1, col. 3, first full paragraph. In other words, the present inventors devised a unique two-step labeling scheme to encode and readout cellular nucleic acid to perform multiplexed error-robust fluorescence *in situ*

hybridization (MERFISH). *See* Chen at Figure 1. This error-robust multiplexed method using encoding and readout probes is known in the art as “MERFISH.” **The claimed subject matter, which includes MERFISH, is distinct from those methods using smFISH probes and provide an advancement over seqFISH (multiplexed methods using smFISH probes) or methods wherein codewords do not “form an error-checking and/or error-correcting code space.”**

EX2054, 16 (emphasis added).

50. In my opinion, the language outlined in the amendment above acknowledges the understanding in the art that 1) the methods of seqFISH were inadequate for the purposes of scaling up the multiplexity of smFISH techniques, 2) that MERFISH was a distinct technique from smFISH, and 3) that MERFISH represented an advancement over seqFISH designed to perform error-robust multiplexed RNA assays beyond the multiplexity limitations of earlier methods. Similar comments are found in related Patent Application No. 18/184,967. During prosecution of that application, the patent applicant stated:

The use of smFISH probes, as taught by each of Femino et al., Lubeck and Cai, and Lubeck 2014, **requires long incubation periods (e.g., overnight) for the smFISH probes to hybridize to the RNA target *in situ*.** *See* Lubeck at “Probe design, Purification and Hybridization” in the Methods section. The primary binding step with the

RNA requires overnight to a few days, as the kinetics of binding are slow. To perform multiplexing from this scheme, the first signal (e.g., a smFISH probe) is removed, a second signal from a second set of smFISH probes are [sic] added to the sample and imaged. **This is not a practical approach to multiplexing if each round of hybridization requires at least an overnight incubation with a new set of smFISH probes.** The present inventors solved that problem by developing a two-step probe binding method, wherein the primary probe is only added in one initial step (which is the longest step and takes more than a day) and then repeated hybridization rounds are performed with secondary probes that bind to the primary probes. This is not a trivial step and greatly enhances the multiplexed FISH methods. Accordingly, the claimed subject matter is a patentably distinct advancement over the cited art and **solved two problems—providing an error-robust coding scheme that enables accurate FISH multiplexing and a shorter method using a two-step probe binding process wherein multiple rounds of hybridization can be performed in a practical time frame.**

EX2055, 17-18 (emphasis added).

51. In my opinion, the remarks above once again indicate that the applicants themselves viewed the MERFISH technique as innovative due to the technique's ability to achieve "accurate FISH multiplexing," whereas previous

techniques “require[d] long incubation periods” and were “not a practical approach to multiplexing.” *See, e.g.*, EX2054, 16; EX2055, 16-18.

52. A POSA would have understood that Chen discloses a home-built MERFISH imaging setup. *See* EX1003, 011 (describing Chen’s setup as comprising a “home-built” wide-field microscope, a “home-built, autofocus system,” and a “home-built fluidics system”). A POSA would also have understood that Chen’s home-built MERFISH setup was intended to image massively multiplexed mRNA in 2D based on Chen’s experimental and setup design. For example, Chen chose to examine human fibroblast cells using a wide-field imaging microscope. EX1003, 011. Chen explains that “[h]uman primary fibroblasts ... are relatively large and flat, facilitating wide-field imaging without the need for optical sectioning” and “z-scanning.” EX1003, 011; *see also* EX1003, 012. As discussed in Section VI.C above, optical sectioning is one method for improving the SNR by removing background fluorescence in otherwise thick samples. *See supra* Section VI.C. Likewise, z-scanning is a technique for imaging in different focal planes along the z-axis of a sample. By choosing to use human primary fibroblasts, Chen could image mRNAs in the sample in a single 2D focal plane without the need to use optical sectioning or z-scanning to improve the SNR. EX1003, 012. This fits with what POSAs would have known about the capabilities of wide-field imaging, namely that wide-field imaging is most useful for obtaining

lateral (x-y) information and less useful for obtaining axial (z-axis) information due to “the lack of axial resolution typical of wide-field imaging.” EX1011, 002; *see also* EX1012, 004 (“wide-field images can provide clear lateral (x- and y-axis) information but only limited axial (z-axis) information”); EX2079, 446 (characterizing “wide-field epifluorescence” as having “[p]oor axial resolution”). Additionally, Chen disclosed that its imaging setup was “configured for oblique incidence excitation.” EX1003, 011. Using oblique incidence excitation on a thin cell sample—such as the flat cells used in Chen—would have eliminated unwanted out-of-focus fluorescence from planes above and below the focal plane. A POSA would have understood this disclosure as a proposed method for removal of out-of-focus fluorescence during 2D imaging. Taking all of this information together, a POSA would have understood that, according to Chen, MERFISH was a highly multiplexed variant of FISH that Chen paired with a 2D imaging setup. Furthermore, a POSA would have recognized that Chen’s focus on multiplexing would create significant challenges for attempts to adapt Chen’s highly multiplexed setup for 3D imaging.

VII. THE 554 PATENT

A. The 554 patent’s claimed system

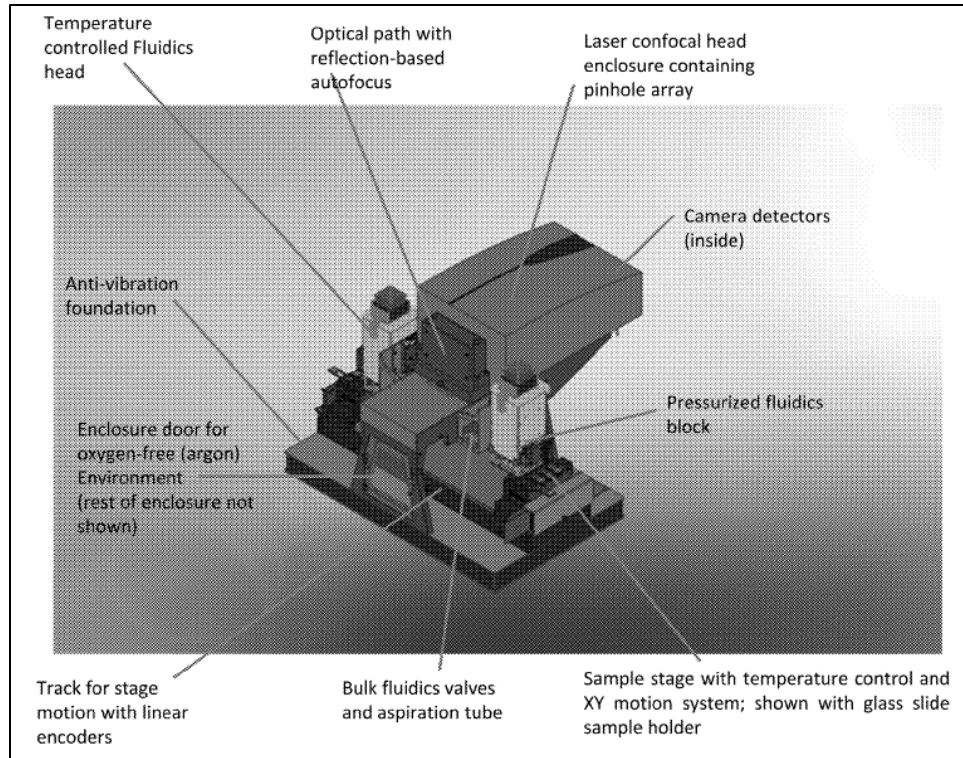
53. The 554 patent is directed to a system for volumetric imaging of individual nucleic acid molecules, such as DNA or RNA, in a sample having a

volume. EX1001, 33:29-34:17. The 554 patent discloses the system as one for volumetric imaging of nucleic acid molecules in a sample down to the scale of 100 nanometers. EX1001, 4:43-50, 16:8-15 (“on the order of 10^{-7} m”). Generally, these claims are directed to a volumetric imaging device “which includes a three-dimensional imaging apparatus, apparatus for fluid exchange, apparatus for temperature control, and apparatus for computational analysis” and which “includes hardware and software functionally assembled.” EX1001, 5:29-33, 16:46-51. The technical subject matter under discussion in this declaration largely involves claim 36 of the 554 patent. EX1001, 33:31-67, 34:1-17.

54. The claimed system combines a mechanical system for optical imaging of nucleic acid molecules, set forth in elements (a)-(d) and comprises specific design components such as a sample holder, temperature control apparatus, XY motion control system, fluidics system, optical system, and a Z-axis motion control system. EX1001, 33:29-34. The claimed system also comprises a computing system for determining the 3D positional information of nucleic acid molecules in an analyzed sample, set forth in element (e). EX1001, 33:55-67, 34:1-17.

55. Figures 2-4 of the 554 patent illustrate an embodiment of the complete all-in-one volumetric imaging system in claim 36. EX1001, FIGs. 2-4. Figure 2 shows the microscope stage with a “[s]ample stage with temperature control and

XY motion system; shown with glass slide sample holder,” which is operatively coupled to the “[s]ample stage with temperature control” and an “XY motion system.” EX1001, FIG. 2. Figure 2 likewise shows “[p]ressurized fluidics block[s]” operationally coupled to a pump in the form of “[b]ulk fluidics valves and aspiration tube.” EX1001, FIG. 2. Figure 3 illustrates an “[o]ptical axis with microscope objective” while Figure 4 also depicts the “[m]icroscope objective” as part of the complete microscopy apparatus. EX1001, FIGs. 3-4. Figure 2 shows an “[o]ptical path with reflection-based autofocus” and a “[l]aser confocal head enclosure containing pinhole array” forming a housing apparatus for the kind of light sources and associated focusing apparatus needed to deliver excitation light to the sample. EX1001, FIG. 2. Likewise, Figure 2 shows light detector elements in the form of “[c]amera detectors” included in the apparatus’ housing. EX1001, FIG. 2. Finally, Figure 4 shows a “Z-axis linear motor with linear encoder feedback for Z motion and Z scanning of objective focal plane through a sample” forming a Z-axis motion control system. EX1001, FIG. 4. Figure 2, which provides a picture of an example system for volumetric imaging, is reproduced below:



EX1001, FIG. 2.

Collectively, these components create a complete, all-in-one system for volumetric imaging of individual nucleic acid molecules.

56. The volumetric imaging system described in the 554 patent provided an early and effective solution to existing challenges in determining 3D positional information of highly-multiplexed nucleic acid molecules. As I discussed above, prior FISH technologies had been limited in their ability to achieve high-multiplexing. *See supra* Sections VI.D.1., VI.D.2. Furthermore, the homebuilt MERFISH setup in Chen was designed with 2D imaging in mind and was not a complete, integrated volumetric imaging system suitable for commercial adaptation. *See supra* Section VI.A (discussing the unsuitability of home-built

microscopy systems for commercial adaptation). The claims of the 554 patent provide both hardware and software designed for successful in situ workflow in a volumetric imaging system for determining 3D positional information of individual nucleic acid molecules. The asserted claims of the 554 patent are practiced commercially by 10x's Xenium Analyzer ("Xenium") and Bruker's/NanoString's CosMx SMI ("CosMx") products. The claimed system of the 554 patent, important to the implementation of these products, is therefore an early integrated, commercially viable bench-top volumetric imaging system unlike the unwieldy home-built setups of the prior art.

B. The 554 patent's prosecution history

57. In preparing this declaration, I have reviewed the prosecution history for the 554 patent and include a brief summary of that history below. I do not claim to be an expert in the patent examination process. I have reviewed the patent prosecution history to develop a general understanding of how the 554 patent came to be issued.

58. I understand that, during examination, the pending claims 9 and 14-38 were initially rejected as obvious over references Keller (EX1042) and Callahan (EX1041). I also understand that pending claims 10-13 were rejected as obvious over Keller, Callahan, and Frisen. EX2006, 537-549. I understand that the Applicant then amended the claims to recite, among other things, that the system

comprises a sample holder configured to receive a substrate comprising a “sample having a volume”; a temperature control apparatus operatively coupled to the sample holder; and a computing system for determining three-dimensional positional information of one or more nucleic acid molecules. EX2006, 671-672.

59. I understand that the Examiner allowed the claims and acknowledged that the prior art was “silent with regard to the computing system as currently claimed.” EX2006, 1275; EX1036, 006. I understand the Examiner also acknowledged that “[o]ther reference of particular interest”—Luo (EX2057) and Green (EX2058)—did not “teach[] the systems as currently claimed, including the computing system configured for determining three-dimensional positional information of the one or more nucleic acid molecules using a plurality of optically-encoded signals.” EX2006, 1275-1276.

VIII. CLAIM CONSTRUCTION

60. I understand that in an IPR, patent claims are given their ordinary and customary meaning as understood by a POSA at the time of the invention (here, November 3, 2015), in view of the patent’s specification and its prosecution history. I also understand that when a patent explicitly defines a claim term in the specification, the patent’s definition controls.

61. In my opinion, a POSA would have understood the phrase “temperature control” in claim 36 (“temperature control apparatus operatively

coupled to said sample holder”) in view of the specification to mean the ability to actively heat and actively cool an apparatus.

62. For example, a POSA would also apply the aforementioned construction of “temperature control” in light of the language from the dependent claims and the patent specification. EX1001, 34:31-32. Dependent claim 43 recites a system in which the temperature control apparatus “comprises a heat sink or a Peltier element,” elements that a POSA would have recognized as being used for cooling parts of a system. EX1001, 34:31-32. Peltier elements are a type of thermoelectric cooler that would have been known to a POSA. A POSA would therefore have understood the inclusion of a Peltier device to signal an active cooling capability as part of the “temperature control” in claim 36. Likewise, the heat sinks of the 554 patent are heat sinks of the kind that provide *active* cooling rather than passive cooling. *See, e.g.*, EX1001, 9:33-35 (“heat sink *in conjunction with a fan*”), 18:12-14 (same), 19:60-62 (same), 26:28-31 (Peltier element and heat sink), 26:35-37 (same), 27:22-23 (“heat sink for Peltier cooler”). The 554 patent’s indication that temperature control could be used in “thermo-cycling for applications such as nucleic acid hybridization, amplification, and sequencing” shows an ability to raise the temperature of a sample. EX1001, 18:5-10. A POSA reviewing this language would have understood that thermocycling techniques—which include common laboratory techniques such as PCR—often “rely on ... an

efficient *heating and cooling system*.” EX2080, 2727. Therefore, it is my opinion that the term “temperature control” in claim 36 requires the capability to actively heat and actively cool.

63. The plain language of the claims is further supported by other disclosures throughout the specification of the 554 patent. For example, Figure 5 depicts a “perspective view of a stage” which includes a “heat sink for the Peltier cooler for stage temperature control” and a “thermo-cycling stage top,” both of which a POSA would have interpreted as indicating that heating *and* cooling systems were involved as part of the claimed invention’s temperature control process. EX1001, FIG. 5, 27:21-23, 27:33-34. Figure 3 also shows “[a] stage for holding samples, with temperature control by Peltier element and heat sink (shown) or liquid cooling is provided.” EX1001, 26:35-37, FIG. 3. The 554 patent also describes using temperature control “such as according to a programmed time and temperature” as in “thermo-cycling for applications such as nucleic acid hybridization, amplification and sequencing”—processes which require both heating and cooling capability. EX1001, 18:5-10. Other language in the specification discloses a “radiator and liquid cooling/circulating system to dissipate heat produced during temperature changes,” “thermoelectric cooling using Peltier elements ... useful with thermo-cycling,” a “heat sink in conjunction with a fan to dissipate heat produced during temperature changes,” and a “radiator and liquid

cooling/circulating system to dissipate heat produced during temperature changes.”

EX1001, 9:35-41, 18:5-7, 18:13-14, 18:15-17. These combined disclosures, which repeatedly highlight the use of Peltier elements, thermo-cycling, and management of temperature by the claimed invention would have reinforced the POSA’s understanding that “temperature control” in the 554 patent referred to actively heating and cooling.

64. Thus, based on the plain language of the claims and the disclosures in the specification, it is my opinion that a POSA would have understood that “temperature control” in the 554 patent referred to the ability to actively raise and lower the temperature. A POSA would have recognized that the “temperature control apparatus” is operatively coupled to the sample holder in the context of claim 36 and therefore must be capable of actively heating and cooling the temperature of the sample holder. EX1001, 31:20-21. This is also the ordinary and customary meaning of the term “temperature control apparatus” in view of the specification – to “control” the temperature of the sample holder, a temperature control apparatus must have the ability to both actively heat and cool the sample holder. An apparatus that can only heat the sample holder—without the ability to cool the sample holder—cannot “control” the sample holder’s temperature as described in the 554 patent, because it is unable to cool the sample holder.

**IX. DR. CZYMMEK FAILS TO DEMONSTRATE THAT THE
CHALLENGED CLAIMS ARE UNPATENTABLE AS OBVIOUS
OVER THE CITED ART**

65. Dr. Czymmek asserts that the claims of the 554 patent would have been obvious over Chen, Shen, and Cai. *See, e.g.*, EX1002, ¶219 (“... to the extent [Chen] is found or argued not to disclose determining 3D positional information, it is my opinion that this was disclosed in Cai and it would have been obvious to modify [Chen] to permit a POSA to use MERFISH to determine three-dimensional positional information about [sic] one or more nucleic acid molecules.”). As I explain below, I disagree with Dr. Czymmek because (i) the art does not teach or suggest a volumetric imaging system for 3D MERFISH, (ii) a POSA would have had no reason to modify Chen’s 2D MERFISH setup (highly multiplexed) for 3D MERFISH imaging in view of Shen or Cai, (iii) Dr. Czymmek does not explain what modifications a POSA would make to Chen in view of Shen or Cai, and (iv) a POSA would have had no reasonable expectation of success in modifying Chen’s 2D MERFISH setup for highly multiplexed 3D MERFISH imaging in view of Cai.

66. In particular, Dr. Czymmek’s combination of prior art does not teach or suggest a system for volumetric imaging comprising a “temperature control apparatus” like that claimed in the 554 patent. Furthermore, Dr. Czymmek does not consider how a POSA would have weighed aspects of fluorescence microscopy—including axial resolution, the point-spread function, the signal-to-noise ratio, and

signal density—when reading and applying the proffered prior art combination.

Additionally, Dr. Czymmek does not discuss how or why the objective in Chen—increasing multiplexity in FISH techniques—would have influenced the POSA’s motivation to combine Chen’s 2D MERFISH setup with the teachings of Shen and Cai. It is my opinion that Chen’s singular objective and focus on increased multiplexing would not have motivated a POSA to modify Chen’s 2D MERFISH setup—in view of Shen and Cai—to create a volumetric imaging system for 3D MERFISH as described in the 554 patent. Even if a POSA were motivated to modify Chen, it is my opinion that a POSA would not have expected a modification for 3D imaging to be successful because the teachings of Chen’s 2D MERFISH setup—increased multiplexity—would conflict with Cai’s light sheet imaging setup without additional modifications—none of which are addressed by Dr. Czymmek.

A. Dr. Czymmek’s argument requires a POSA to modify Chen’s 2D MERFISH setup to 3D MERFISH

67. Dr. Czymmek’s argument is founded on the premise that a POSA reading Chen, Cai, and Shen would have been motivated to convert Chen’s home-built 2D MERFISH imaging setup, in view of Cai and Shen, into a modified version of Chen’s imaging setup that performs 3D MERFISH imaging. For example, Dr. Czymmek asserts that “a POSA ... would have expected success in using the system to perform MERFISH as taught in [Chen]” and that “it would

have been obvious to modify [Chen] with Cai’s teachings using multiple light sources” EX1002, ¶¶153, 170. Dr. Czymmek also asserts that “a POSA interested in MERFISH” would seek “application of Z-axis scanning and imaging to [Chen]’s MERFISH protocol,” that “a POSA would have been motivated to use the MERFISH method for 3D investigation,” and that “it would have been obvious to modify [Chen] to permit a POSA to use MERFISH to determine three-dimensional positional information.” EX1002, ¶¶186, 191, 207, 219, 305; *see also* EX1002, ¶312 (“a POSA interested in operating [Chen]’s automated system for MERFISH imaging ...”).

68. I understand from Dr. Czymmek’s statements that his argument is predicated on modifying Chen’s 2D MERFISH imaging setup to arrive at the volumetric imaging system claimed in the 554 patent. However, it is my opinion—as further outlined below—that Dr. Czymmek’s theory is a misapplication of the prior art that does not accurately consider a POSA’s understanding and motivation as of November 3, 2015.

B. Dr. Czymmek’s art combination of Chen, Shen, and Cai fails to teach or suggest a system for volumetric imaging comprising a temperature control apparatus as understood by a POSA

69. As discussed in preceding sections, the volumetric imaging system claimed in the 554 patent must comprise a temperature control apparatus, and temperature control—as understood by a POSA from the 554 patent’s

disclosures—requires the ability to actively raise *and lower* temperature. Dr.

Czymmek argues that the “Bioptechs FCS2 chamber” disclosed in Chen “would have been known to include a temperature control capability.” EX1002, ¶142. I disagree with Dr. Czymmek’s opinion for several reasons outlined below.

70. First, there is no evidence that Chen’s “FCS2 flow chamber” has associated temperature control. EX1003, 011. Chen does not disclose using any temperature control associated with the FCS2 flow chamber. EX1003, 011. Instead, Chen only discloses that an FCS2 flow chamber was used in performing the MERFISH protocol alongside an objective heater. EX1003, 011. Dr. Czymmek asserts that “the use of a temperature control elements [sic] with the flow chamber was well-established in the art” and cites to Sivaramakrishnan as providing disclosure of an “FCS2 chamber” that “maintains ... cells and media at 37°C.” EX1002, ¶142. I note that Sivaramakrishnan used an FCS2 flow cell for imaging live cells, and thus needed to maintain the FCS2 flow cell at 37°C to keep the cells alive. *See* EX1007, 004 (discussing that the “Bioptechs FCS2 chamber maintains the cells and media at 37°C”); *see also* EX2081, 5 (discussing that maintaining temperature for live cell imaging “ensure[s] that observed cell dynamics are an accurate representation of in vivo cell behavior” and that “the most commonly used temperature is 37°C for mammalian cell lines.”). In contrast, Chen’s 2D MERFISH experiments imaged *fixed cells*, which do not need to be maintained at

37°C. EX1003, 011; *see* EX2052, 13 (noting that a hybridization buffer was added to “room temperature samples” composed of “[f]ixed yeast cells”).

71. Even if Chen did teach or suggest using an FCS2 flow chamber capable of heating and maintaining 37°C, a POSA would not have understood the system to constitute a “temperature control apparatus,” as it would only be capable of raising temperature above ambient temperature, not actively lowering temperature below ambient temperatures. In his declaration, Dr. Czymmek asserts that “it was well-known to a POSA that the Biopetechs FCS2 flow cell includes temperature control.” EX1002, ¶143. Dr. Czymmek appears to base his opinion on the teachings in Sivaramakrishnan. However, Sivaramakrishnan indicates that the “temperature control” of the FCS2 chamber is designed for *heating* and maintaining *heat*. *See, e.g.*, EX1007, 004 (disclosing an “FCS2 chamber” that “maintains the cells and media at 37°C”). Furthermore, the “FCS2 instruction document” discloses an FCS2 controller exclusively capable of raising temperature and maintaining the temperature. EX1006, 001. The document instructs the user that “[w]hen the controller is turned on ... it will read the ambient temperature, then slowly *begin to rise* until it reaches setpoint temperature.” EX1006, 004. This instruction therefore teaches that the associated FCS2 flow chamber is only capable of increasing and then maintaining the temperature of a sample compared to ambient temperature. Thus, at most, Chen’s disclosed FCS2 chamber only raised

the temperature. Accordingly, Dr. Czymmek's combination of art did not teach or suggest a "temperature control apparatus" as claimed in the 554 patent.

72. Dr. Czymmek also asserts that a POSA would have understood that the objective heater disclosed in Chen was an additional temperature control system per claim 36. EX1002, ¶144. However, it is my opinion that a POSA would have understood that the "objective heater" disclosed in Chen is merely a heating element rather than a "temperature control," as claimed. EX1003, 011. For example, the instructions for the FCS2 controller indicate that the "Bioptechs Objective Heater" of the FCS2 comprises a "heating band and temperature sensor" and includes no mention of any cooling capacity associated with the objective heater. EX1006, 006. Thus, Chen's disclosure of an "objective heater" does not teach or suggest the "temperature control apparatus" element to a POSA, as it only discloses the ability to raise temperature via the objective lens heater. EX1006, 006.

73. Furthermore, I disagree with Dr. Czymmek's opinion that the objective heater is "operatively coupled to the sample holder," as required in claim 36(a)(ii). The FCS2 controller instructions teach that the objective heater "plugs into a separate Temperature Controller which regulates the objective temperature within 0.1°C of SETPOINT." EX1006, 006. This description would teach a POSA that the objective heater in Chen was operatively coupled to the microscope

objective (the objective being an element recited in claim 36(c)(i)) and a separate objective temperature controller, but not operatively coupled to the sample holder as required by claim 36(a)(ii).

74. Neither Cai nor Shen teach the temperature control element of claim 36. Shen's only disclosure for temperature in fluorescence microscopy is an acknowledgement that thermal fluctuations can affect microscope focus in extended time-lapse studies. EX1032, 001. Cai discloses performing enzymatic reactions at room temperature or 37°C. EX1003, 27:20-23, 55:43-45, 55:64-66. Thus, neither Cai nor Shen teach a system for volumetric imaging comprising an apparatus capable of actively heating and cooling the sample holder, as required by claim 36.

75. Taken together, the disclosures discussed above would not teach a POSA to modify Chen's 2D MERFISH setup with a temperature control apparatus capable of both actively heating and cooling. At most, the cited art teaches various methods of maintaining sample temperature via heating without any teaching of an associated cooling capability.

C. Dr. Czymmek's prior art combination fails to teach or suggest a volumetric imaging system for determining three-dimensional positional information of one or more nucleic acid molecules as claimed in the 554 patent

76. According to Dr. Czymmek, a POSA would have been motivated to modify Chen's 2D MERFISH imaging setup in view of Shen and Cai's disclosures

to create a volumetric imaging system for 3D MERFISH. *See, e.g.*, EX1002, ¶¶153 (“Thus, in my opinion, a POSA would have recognized that the same modifications to the same system can be made the same way, and would have expected success in using the system to perform MERFISH as taught in [Chen]”), 170 (“it is my opinion that it would have been obvious to modify [Chen] with Cai’s teachings using multiple light sources for the benefit of obtaining greater diversity of signals for generating distinct barcodes”), 207 (“it is my opinion that a POSA would have been motivated to use the MERFISH method for 3D investigation and would have looked to the relevant teachings in Shen and Cai regarding Z-axis motion control that provides for 3D imaging”), 219 (“to the extent [Chen] is found or argued not to disclose determining 3D positional information, it is my opinion that this was disclosed in Cai and it would have been obvious to modify [Chen] to permit a POSA to use MERFISH to determine three-dimensional positional information about [sic] one or more nucleic acid molecules”).

77. Thus, Dr. Czymmek’s theory is that a POSA would be motivated to modify Chen’s 2D MERFISH imaging setup in view of Cai and Shen’s disclosures to create a volumetric imaging system for determining the 3D positional information of individual nucleic acid molecules. As explained below, I disagree with Dr. Czymmek’s conclusion. It is my opinion that a POSA would have no reason to modify Chen’s 2D MERFISH imaging arrangement into a volumetric

imaging system for determining three-dimensional positional information of individual nucleic acid molecules based on Dr. Czymmek's relied-upon disclosures in Cai and Shen.

1. Neither Chen nor Shen suggest to a POSA a system for determining three-dimensional positional information of one or more nucleic acids

78. Neither Chen nor Shen suggests a system for determining three-dimensional positional information for one or more nucleic acid molecules. For example, Chen's 2D MERFISH setup used "wide-field imaging with oblique-incidence illumination, without optical sectioning and z-scanning." EX1003, 012. A POSA would have known that wide-field imaging is a technique that produces predominantly 2D data due to the lack of axial resolution in wide-field imaging. EX1011, 002; EX1012, 004; EX2079, 446. Shen summarizes methods of autofocusing microscope lenses, including methods in which "hardware acquires a z stack of images for each focus attempt" that is subsequently used to calculate focus indices and the ideal position of the microscope lens in relation to the sample. EX1032, 002, 006. A POSA would have understood this process to be a method for enhancing 2D imaging quality in which "[t]he sharpest image is acquired at the best focus position calculated from the focus indices," rather than being a method to obtain 3D positional information. EX1032, 002.

79. Further, as I describe below, it is my opinion that the underlying goals

of Chen, the lack of 3D imaging in Chen, and the 2D nature of Shen's imaging setup would not suggest or teach a POSA a system for determining the three-dimensional positional information of one or more nucleic acids.

a. A POSA would have understood that Chen's stated goal and the benefit of MERFISH was increased 2D multiplexing, not determining 3D positional information

80. Prior to Chen, predecessor methods to MERFISH (smFISH and seqFISH) were limited by their multiplexing capability—namely, the number of RNA species that the techniques could simultaneously measure in a single cell. EX1003, 002. In this context, a POSA would have understood that Chen's overall goal in developing MERFISH, a “highly multiplexed smFISH imaging method” that “substantially increases the number of RNA species that can be simultaneously imaged in single cells,” was to create a technique that overcame the limits on multiplexity that constrained predecessor techniques. EX1003, 002. This understanding is reflected by Chen, as Chen explains that “the high cost of the massive number of distinct FISH probes needed and the long time required to complete many rounds of hybridization” in existing smFISH and seqFISH methods “made it difficult to probe a large number of RNA species.” EX1003, 002. Chen further disclosed that the investigators “sought to further increase the throughput of [their] MERFISH measurement by simultaneously imaging ~ 1000 RNA species.” EX1003, 007. When suggesting further steps, the authors suggested additional

modification to “further increase the number of detectable RNA species.” EX1003, 008. The authors also discussed some challenges associated with increasing degrees of multiplexity, noting that some of the most highly expressed genes in the transcriptome would remain outside of the reach of their imaging setup even following further improvements. EX1003, 009. Finally, the abstract of the article focuses on the multiplexity of MERFISH and the potential benefits of said multiplexity. EX1003, 002. In particular, the abstract emphasizes MERFISH as an approach capable of imaging “thousands of RNA species” with a demonstrated ability to image “100 to 1000 distinct RNA species in hundreds of cells.” EX1003, 002.

81. A POSA would also have understood from Chen that achieving increased multiplexing through combinatorial labelling would face a tradeoff—namely, that “detection errors also increase exponentially” alongside an increase in detectable RNA species, ultimately requiring some form of error correction ability to maintain accuracy. EX1003, 001; *see also* EX1003, 002 (discussing challenges associated with combinatorial labelling of RNA). Therefore, a POSA would understand that the more multiplexing is increased, the more error correction is needed. Chen explicitly explains that “not only does the number of addressable RNA species increase[]” with the increased number of distinct signals per RNA species, but “the detection error rate also grow[s] exponentially” with the number

of distinct signals. EX1003, 002. Chen describes this as a “substantial challenge” to “scaling up the throughput of smFISH” through combinatorial labelling.

EX1003, 002. A POSA reading Chen therefore would have understood Chen to teach away from the kind of smFISH combinatorial labelling technique described in Cai.

82. Based on the information above, it would have been clear to a POSA that the objective of Chen’s research was to increase multiplexing to solve existing, recognized challenges related to the limited multiplexity of smFISH. *See, e.g.*, EX1003, 002 (citing EX2051 and EX2052). In fact, this understanding would remain the case in subsequent years, when publications by the research group in Chen continued to highlight the then-existing challenges to increasing multiplexity in MERFISH. *See* EX1021, 002 (highlighting that “even the most advanced multiplexing efforts have only extended this approach to the simultaneous measurement of ~10-30 RNA species”). The same group also later reported “an improved MERFISH method that drastically increases the throughput of this technique,” and disclosed “increase[ing] the throughput of [MERFISH] by two orders of magnitude.” EX2053, 11047, 11050. Therefore, it is my opinion that a POSA interested in performing MERFISH would have been interested in harnessing the touted multiplexing capabilities of MERFISH.

83. Taken together, Chen’s abstract, the identified problem to be solved,

the described benefit of the outlined MERFISH setup, the discussion of remaining limitations on multiplexity, and the future directions of Chen all suggest to a POSA that Chen's ultimate goal is increased multiplexity through MERFISH. *See* EX1003, 002, 007-008.

b. Consistent with Chen's multiplexing goal, a POSA would have understood that Chen disclosed a setup for performing MERFISH imaging in two-dimensions

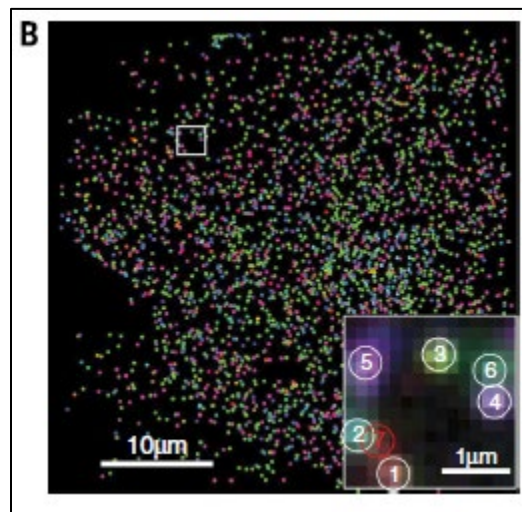
84. A POSA would have understood that several of the design decisions in Chen's MERFISH setup were tailor-made for 2D imaging of nucleic acids in cells. For example, a POSA would have understood Chen's MERFISH setup to be intended for 2D imaging based on Chen's use of traditional "wide-field imaging." EX1003, 012. As explained in Section VI.D.1, traditional wide-field imaging was an imaging system well known in the art for poor axial resolution that rendered it inadequate for measuring 3D coordinates. *See, e.g.*, EX1012, 004 ("wide-field images can provide clear lateral (x- and y- axis) information but only limited axial (z-axis) information."); EX1011, 002 (disclosing that wide-field imaging data are in 2D due to "lack of axial resolution typical of wide-field imaging."); EX2079, 446 (characterizing "[w]ide-field epifluorescence" as having "[p]oor axial resolution."). Given that traditional wide-field imaging was known to have poor axial resolution, a POSA would have understood that Chen's use of wide-field imaging for MERFISH indicated a focus on 2D imaging in Chen's imaging

arrangement.

85. Moreover, Chen discloses that the MERFISH setup images a layer of human primary fibroblasts that are “relatively large and flat.” EX1003, 011. A POSA would have understood that the majority of targeted cellular RNAs and fluorescent signals on this flat layer of cells would be found in a single focal plane. EX1003, 012. Chen refers to this focal plane as the “basal focal plane.” EX1003, 012. Chen further explains that human primary fibroblasts were chosen because the cell type is “relatively large and flat, facilitating wide-field imaging without the need for optical sectioning.” EX1003, 011. As explained in Section VI.C above, optical sectioning was a well-known method in the art for removing fluorescence contributions by out-of-focus light in an image plane. *See supra* Section VI.C. A POSA would have applied this understanding and known that Chen’s disclosure indicated that the MERFISH technique was designed for imaging mRNA in a single 2D focal plane (the “basal focal plane”) without the need to remove background fluorescence from planes above and below the focal plane on the z-axis. Additionally, Chen disclosed that its 2D MERFISH imaging setup was “configured for oblique incidence excitation.” EX1003, 011. The use of oblique incidence excitation on a thin cell sample, such as Chen’s flat cells, would have kept out-of-focus fluorescence from the focal plane by eliminating unwanted excitation in other planes. Thus, Chen’s explicit teaching that the MERFISH

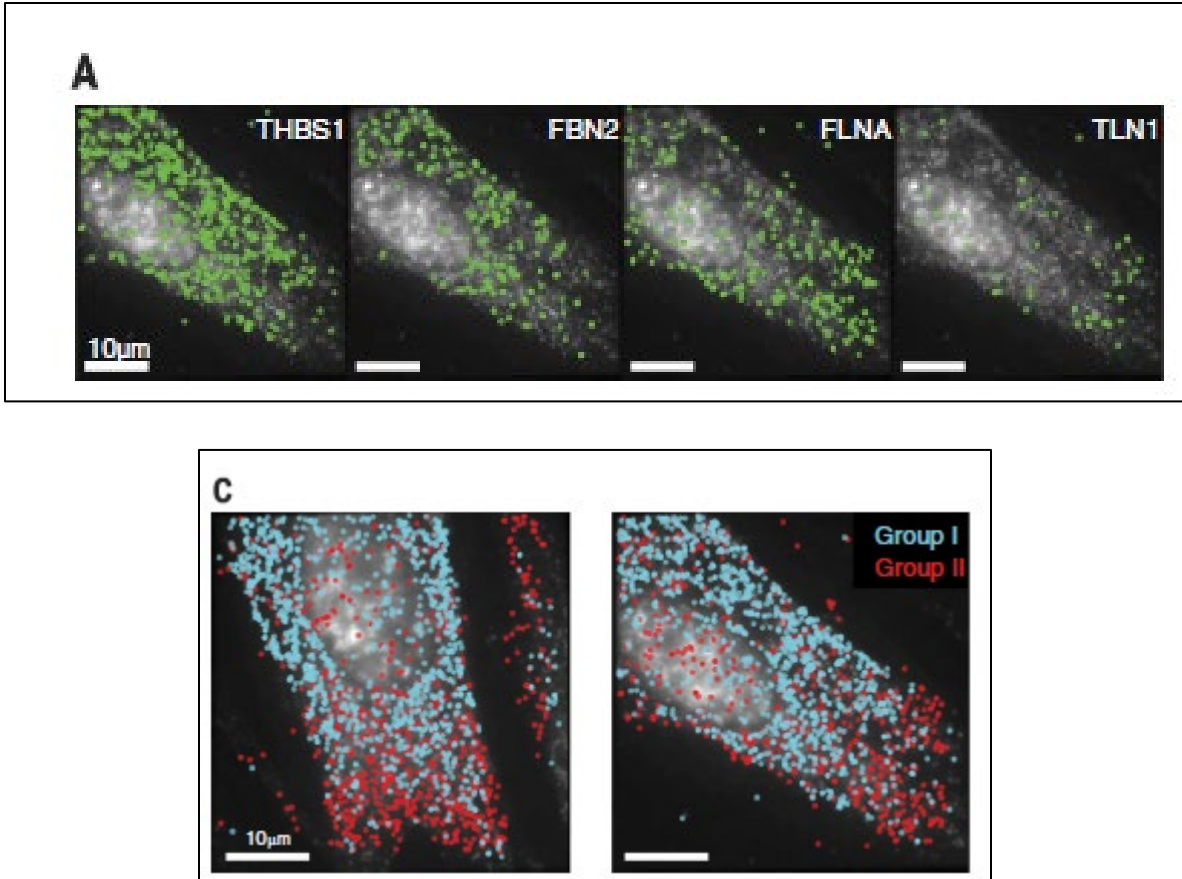
imaging setup operated effectively “without the need for optical sectioning” would have dissuaded a POSA from applying the MERFISH method to more than one plane along the z-axis. EX1003, 011.

86. Moreover, the reported data described in Chen would have confirmed to a POSA that Chen’s MERFISH setup was applicable for 2D imaging. *See, e.g.*, EX1003, 004-005, 011-012, FIGs. 2, 4, 5. For example, Figure 2B in Chen (shown below) shows the localization of individual RNA molecules, shown as fluorescent spots, after 16 hybridization rounds in a 2D visual representation.



EX1003, FIG. 2B.

Figures 4A and 4C likewise show two-dimensional “spatial distributions” of RNA (RNA species and two RNA groups, respectively) as 2D figures showing images of cells overlaid with fluorescent spots representing the location of RNA molecules.



EX1003, FIGs. 4A, 4C.

In “mapping the spatial distributions of RNAs,” Chen discloses that the 2D spatial location of the molecules includes “RNA transcripts enriched in the perinuclear region, some enriched in the cell periphery, and some scattered throughout the cell,” all of which are represented in Figures 4A and 4C as fluorescent spots on a single layer of flat fibroblast cells. EX1003, 005, FIG. 4. Taken together, these fluorescent images solely depict fluorescence imaging from Chen’s MERFISH setup in a 2D format. A POSA viewing these disclosures would have understood that the primary output of MERFISH was 2D localization data for RNA, as the

data and figures presented do not indicate any attempt or intention to apply MERFISH to 3D imaging.

87. Moreover, the analysis of imaging data described in Chen would have indicated to a POSA that Chen's 2D MERFISH setup was intended for 2D, not 3D imaging. Chen discloses that the fluorescent spots in the figures were "identified and localized in each image by using a multi-Gaussian-fitting algorithm [] assuming a Gaussian with a uniform width of 167 nm." EX1003, 012. A POSA would have understood this disclosure to indicate that Chen's setup uses a Gaussian function that only considers the "width" parameter ("167 nm") to localize RNA molecules in each image. EX1003, 012. Furthermore, "the number of each RNA species was counted in individual cells within each 40- by 40- μ m imaging area," and the authors acknowledged that "[t]his number accounts for the majority but not all RNA molecules within a cell *because a fraction of the cell could be outside the imaging area or focal depth.*" EX1003, 012. A POSA would have understood that the application of a Gaussian function to adjacent 2D image areas (the "40- by 40- μ m imaging areas" described above) teaches a setup for determining 2D information for imaged molecules rather than 3D information, as the authors only contemplated the use of x- and y-axis information for the molecules within these imaging areas.

88. Dr. Czymmek suggests that "a POSA in possession of [Chen's]

disclosures would recognize that MERFISH has the capability for three-dimensional spatial positioning” because “[Chen] points to this kind of three-dimensional analysis in describing optically sectioning cells ‘by collecting stacks of images at different focal depths through the entire depth of the cells.’” EX1002, ¶204 (citing EX1003, 012). I disagree with this opinion, as it mischaracterizes the nature of Chen’s disclosure. The statement Dr. Czymmek cites does not represent a three-dimensional analysis based on MERFISH. Instead, it relates to a conventional smFISH-based (i.e., *not* the highly multiplexed MERFISH technique) quality-control experiment that confirms there was no need to image the cell sample in focal planes other than the basal focal plane used by Chen’s 2D MERFISH setup. EX1003, 011, 012. Chen explains that the authors performed the smFISH quality-control experiment to “quantif[y] the fraction” of individual RNA species that were outside of the basal focal plane targeted by Chen’s 2D MERFISH setup, and this quality control experiment was performed precisely because the MERFISH setup imaged fibroblasts “without optical sectioning and z-scanning.” EX1003, 012. Thus, a POSA would have understood that Chen disclosed quantifying the fraction of RNA molecules that were outside of the basal focal plane targeted by Chen’s setup to confirm that the setup actually captured the majority of the relevant fluorescent RNA signals without imaging additional planes. EX1003, 012. Chen expressly concludes that “only a small fraction” of the

RNA molecules were “outside the imaging range of a fixed focal plane” targeted by Chen’s MERFISH setup, ultimately confirming that—as predicted by the authors—optical sectioning and z-scanning were unnecessary for Chen’s 2D MERFISH approach. EX1003, 011, 012. Dr. Czymmek also admits that this statement in Chen is “in connection with its smFISH validation experiments.” EX1002, ¶¶128, 183. Once again, this would suggest to a POSA that 3D imaging was not a contemplated option for MERFISH.

89. Chen further states that “[f]rom an imaging perspective, any optical sectioning technique could be used in MERFISH to allow the imaging of RNAs in thicker cells or tissues.” EX1003, 012. As explained earlier in Section VI.C, a POSA would have known that optical sectioning is a technique that allows for removal of out-of-focus fluorescence and helps to enhance contrast and improve image quality. *See supra* Section VI.C. Chen’s disclosure here simply suggests the use of optical sectioning to remove background fluorescence arising from planes above or below the 2D focal plane of the MERFISH setup in thick samples. A POSA would have known that wide-field imaging, even with oblique incidence excitation, illuminates planes above and below the focal plane. For example, Chen itself disclosed that its “excitation geometry illuminated the full depth of [the] cells” in the MERFISH imaging setup. EX1003, 012. Because cells or tissues thicker than the flat fibroblast layer of Chen would experience illumination of

several additional planes, imaging these thicker cells would lead to higher out-of-focus fluorescent signals in the 2D focal plane, increasing background and thereby decreasing the SNR. *See supra* Section VI.C. Accordingly, A POSA would have understood that Chen’s discussion of fibroblast cells and optical sectioning suggested a 2D application of MERFISH rather than using MERFISH to determine 3D positional information of individual nucleic acid molecules.

90. Accordingly, a POSA would *not* have understood Chen to teach or suggest using the MERFISH arrangement to determine 3D positional information of one or more nucleic acid molecules. Therefore, Chen does not teach the element in claim 36(e) of the 554 patent.

c. Shen disclosed an imaging system designed for 2D imaging

91. Dr. Czymmek offers Shen as an example disclosure of “an automated X-Y motor control system” and for teaching “‘various solutions’ for ‘micropositioning the objective lens in its axial, or z position.’” EX1002, ¶148, ¶191. However, Dr. Czymmek does not rely on Shen to teach the “determining 3D positional information” element of claim 36. In my opinion, this is because Shen does not disclose such a teaching. Shen instead discloses methods of autofocusing microscope lenses, including methods in which “hardware acquires a z stack of images for each focus attempt” that is subsequently used to calculate focus indices and the ideal position of the microscope lens in relation to a sample. EX1032, 002,

006. Shen then discloses selecting “the sharpest image” at “the best focus position calculated from the focus indices,” and fitting the “best focus position” to a 2D Gaussian curve. EX1032, 002, 006. Finally, Shen discloses that the automated focus method is based on “two-dimensional spatial resolution.” EX1032, 005.

When examining Shen’s disclosures in their entirety, a POSA would have understood Shen to describe a method for enhancing 2D image quality by automatically selecting the sharpest image among several z-stacked images. Shen only considers images from a z stack to determine the sharpest image amongst them, thereby selecting a single focus plane (a 2D image) for the final output. Thus, Shen does not suggest determining the 3D positional information of nucleic acid molecules.

2. Dr. Czymmek’s relied-upon disclosures in Cai do not show that Cai teaches or suggests modifying Chen’s setup to arrive at a volumetric imaging system for determining 3D positional information of one or more nucleic acid molecules

92. Dr. Czymmek asserts that “it would have been obvious to modify [Chen] in view of Cai” to “permit a POSA to use MERFISH to determine three-dimensional positional information about [*sic*] one or more nucleic acid molecules.” EX1002, ¶205, ¶219. However, I note that Dr. Czymmek does not explain the particular modifications that a POSA would have made to actually convert Chen’s 2D MERFISH imaging setup into a volumetric imaging system for

determining three-dimensional positional information of nucleic acid molecules per the teachings in Cai. Dr. Czymmek asserts that “Cai teaches using seqFISH to profile nucleic acid molecules and obtain ‘three dimensional spatial information’” (EX1002, ¶206, citing EX1004 at 21:50-51) and that “Cai teaches the claimed Z-axis motion control system to permit three-dimensional interrogation.” (EX1002, ¶206). However, even if a POSA were motivated by Cai to modify Chen’s 2D MERFISH arrangement for 3D imaging (and in my opinion, they would not have been so motivated), the POSA would have required more than a mention of the “Z-axis motion control system” that Dr. Czymmek claims is taught by Cai. For the reasons explained below, I disagree with Dr. Czymmek’s conclusions.

a. A POSA would have understood that Cai’s imaging setup is of a different design and function from Chen’s home-built MERFISH setup

93. Dr. Czymmek bases his argument in favor of modification on three purported teachings in Cai: (i) a purported z-axis control system for a light sheet microscope depicted in Cai’s Figure 17, (ii) Cai’s disclosure of using two or more fluorescent colors, and (iii) Cai’s sentence referring to “three-dimensional spatial information.” EX1002, ¶86-87 (citing EX1004, FIG. 17), ¶279, ¶206. In my opinion, none of the aforementioned disclosures would have motivated a POSA to modify Chen’s home-built 2D MERFISH imaging setup to create a volumetric imaging system for determining three-dimensional positional information of

nucleic acid molecules. Instead, a POSA would have understood that Chen and Cai used completely different systems that were not interchangeable due to their differing geometries, intended uses, and their optimizations for imaging different cell types.

94. A POSA would have known that the light sheet microscope imaging system in Cai was completely different from Chen's 2D MERFISH imaging setup and was not designed for use with MERFISH. Cai disclosed that the light sheet microscope used two mirrors and a "cylindrical lens" to generate a "plane of light sheet" which "illuminates the sample from the side, and is perpendicular to the objective." EX1004, 47:31-40. A POSA would have generally known that light sheet microscopes like that demonstrated in Figure 17 of Cai use large working distances and lower NA to account for the optical machinery and specific physical geometry required to create the perpendicular plane of light and accommodate the larger specimens being imaged. *See* EX1004, FIG. 17. For example, "perpendicular excitation/detection geometry required by LSFM [light sheet fluorescence microscopy] forces the use of long working distance, somewhat *low NA* (typically 1.0 or less) objective lenses." EX1019, 007. In line with this, Cai describes such a geometry for its disclosed microscope system. For example, Cai discloses that its light sheet microscope system provided "large FOV [field of view] imaging ... with 20x low NA objectives." EX1004, 44:34-35. Other

disclosures in Cai also fall in line with the description of such a geometry. *See* EX1004, 47:52-53 (“20x water immersion objective”), 42:17-19 (“In some embodiments, a single field of view (FOV) on a microscope is 0.5 mm×0.5 mm×2 μm with a 20x air objective”), 43:29-35 (“In some embodiments, a light sheet is generated using a cylindrical lens to focus one axis of the beam into an about 10 μm height and an effective width or FOV of about 200 μm”). As explained above, a POSA would have understood that a disclosed system with such a large field of view and *low NA* was not designed for single-molecule detection in the context of highly multiplexed RNA imaging, especially since Cai’s light sheet images only show fluorescence imaging for a single RNA species. *See supra* Section VI.B; *see also* EX1004, FIGs. 21-23.

95. Cai also disclosed implementing a “hybridization chain reaction” (HCR) “to amplify [the] FISH signal” in order to use the large field of view and low NA objective light sheet imaging system to effectively image the FISH signal. EX1004, 43:29-35. Cai describes HCR as “[a]mplification of FISH signals allows large [field of view] imaging of brain slices with 20x low NA objectives” to “increase the signal-to-background” of smFISH. EX1004, 44:34-40. Cai discloses that this signal amplification occurs by using “nucleic acid probes complementary to mRNA targets,” which bind to the target RNAs and “trigger chain reactions in which metastable fluorophore-labeled nucleic acid hairpins self-assemble into

tethered fluorescent amplification polymers.” EX1004, 44:40-44. Therefore, Cai’s HCR system generates a large fluorescent polymer at the site of the nucleic acid molecule, which increases a molecule’s signal to compensate for the large field of view and small NA of the microscope objective. A POSA would have understood that this HCR application represented an attempt to mitigate the negative consequences of applying a low resolution imaging technique to signals from a technique with “the high detection efficiency of smFISH.” EX1004, 43:34-35.

96. A POSA would have understood that Chen’s 2D MERFISH imaging setup was fundamentally different to the system disclosed in Cai. Chen discloses that its setup uses a “1.45 NA, 100x oil immersion objective.” EX1003, 011. A POSA would have understood that this disclosed system was designed with a *high NA* (the 1.45 NA of the objective lens) and small field of view (from the high magnification of the 100x lens) that would have enabled significantly higher resolution and signal collection than the system disclosed in Cai, at the cost of field of view. A POSA would have been aware of this tradeoff between resolution and field of view from the art. For example, a POSA would have been aware that “high NA also comes at the expense of reduced working distance” and “longer working distances for objective lenses ... comes at the cost of lowering the numerical aperture.” EX1012, 002; EX1019, 002. It is my opinion that a POSA would have applied this knowledge of the tradeoff in resolution and field of view and

understood that Chen's setup—disclosed as possessing a *high NA* and *small field of view*—would have a higher resolution suited to imaging the high-density signals from the MERFISH platform. In contrast, a POSA would have understood that Cai's system—possessing *low NA* and *large field of view*—was more suited to lower resolution imaging of wider image planes. Furthermore, Chen's imaging setup was designed for massive multiplexing of over 1,000 RNA species simultaneously, as compared with Cai's setup for imaging single molecules— β -actin mRNA—after amplifying the RNAs. EX1003, 001-002; EX1004, 47:30-56. Therefore, Chen's imaging setup was for highly multiplexed, simultaneous imaging of thousands of RNA species at high NA and small field of view, whereas Cai's arrangement was designed for imaging single RNA species at low NA and large field of view. A POSA would have recognized that Cai's low NA and high field of view system has a completely different design and function compared to Chen's high NA, low field of view MERFISH setup.

97. Moreover, a POSA would have understood that Cai's light sheet system was specifically designed with the refractive index of CLARITY cleared brain tissue samples in mind rather than the fibroblasts of Chen's 2D MERFISH setup. It was generally known at the time that matching the refractive index of an objective lens with the refractive index of the sample was important to ensure image quality and minimize imaging complications in fluorescence microscopy.

For example, Chung specifically highlights in the context of CLARITY imaging that “it will be important for optics to be refined and for objectives to be developed that are *matched to the refractive index of clarified tissue* to minimize aberration and maintain both high resolution and long working distance.” EX2059, 510. This is further demonstrated by Fischer, where the authors note that “[r]efractive mismatches between thick specimens, glass, and immersion media further add to spherical aberration, inducing errors in Z localization.” EX1019, 002. With this general knowledge in mind, a POSA would have understood that Cai’s system was specifically engineered so that the refractive index of the objective was specifically tailored to thick CLARITY samples. Furthermore, the POSA would not have viewed this system as interchangeable with the setup in Chen, where the 2D MERFISH method was designed to image thousands of nucleic acids in “relatively large and flat” fibroblasts using a high NA 100x objective lens that would require a different refractive index. EX1003, 011.

98. Accordingly, a POSA would have understood that Cai and Chen disclosed distinct imaging arrangements that were not interchangeable due to their conflicting geometries, intended uses, and their adaptations to different target cells.

b. Dr. Czymmek does not articulate any viable reasons to modify Chen’s setup in view of Cai

99. Dr. Czymmek’s obviousness argument relies on three purported teachings in Cai to show that a POSA would have combined Cai’s system with

Chen's homebuilt MERFISH setup: (i) Cai's disclosure of a z-axis motion control system; (ii) Cai's disclosure of using two or more fluorescent colors; and (iii) Cai's single sentence referring to "three-dimensional spatial information." EX1004, 21:50-51. Contrary to Dr. Czymmek's opinions, the aforementioned disclosures would not have motivated a POSA to modify the 2D MERFISH setup in Chen in view of Cai to create a volumetric imaging system for determining 3D spatial information of one or more nucleic acid molecules.

100. First, Chen's setup already had a "home-built, autofocusing system," and Dr. Czymmek does not provide an explanation as to why a POSA would have modified this setup with a different autofocus arrangement. EX1003, 011. Dr. Czymmek first asserts that the POSA would have "been motivated to combine the teaching in Shen with [Chen's] system because [Chen] suggests such a combination." EX1002, ¶186. Moreover, Dr. Czymmek argues that "a POSA interested in MERFISH would have looked to sources such as Shen to determine specific ways to build an autofocusing system in an Olympus IX-71 microscope." EX1002, ¶186. However, Dr. Czymmek does not explain why a POSA would be motivated to modify the MERFISH setup in Chen to use a different autofocusing system when Chen already outlines an autofocusing system. *See* EX1003, 011 (disclosing that "[c]onstant focus was maintained throughout the imaging process with a home-built, autofocusing system.").

101. Second, Cai's purported disclosure of using "two or more light sources" would not have been useful to a POSA reading Chen and interested in practicing MERFISH. Dr. Czymmek first argues that "[Chen] discloses the use of two or more light sources (*i.e.* three lasers) and the two or more light sources are configured to emit two or more colors of light," relying on Chen's disclosure of solid state lasers for excitation of the "Cy5-labeled readout probes, the fiducial beads, and nuclear counterstains." EX1002, ¶168; EX1003, 011. In my opinion, this is not a disclosure of "two or more light sources" as claimed in the 554 patent. EX1001, 33:47-48. A POSA would have understood that the "two or more light sources" in claim 36 were "configured to emit two or more colors of light," and that these colors would be "associated with one or more nucleic acid molecules" per claim 36(e). EX1001, 33:45-49, 33: 65-67. In Chen, only one of the colors of light—emitted by the Cy5 dye attached to the readout probes—is associated with imaged RNA molecules, while the other colors of light would be associated with the fiducial beads and nuclear counterstains. EX1003, 011 ("Illumination was provided at 641, 561, and 405 nm by using solid-state lasers (MPB communications, VFL-P500-642; Coherent, 561-200CWCDRH; and Coherent, 1069413/AT) for excitation of our Cy5-labeled readout probes, the fiducial beads, and nuclear counterstains, respectively."). Therefore, the fluorescence tied to the fiduciary bead and nuclear counterstain are not associated with one or more nucleic

acid molecules and do not meet the element of claim 36(e).

102. Dr. Czymmek further argues that “it would have been obvious to modify [Chen] in view of Cai to arrive at the use of multiple colors” to increase MERFISH’s multiplexing capabilities. EX1002, ¶213. However, Chen had already suggested the use of “two-color imaging in each round” as an alternative method to “increase the throughput of [their] MERFISH system.” EX1003, 007. Chen in fact made this alteration one year later when they reported improvements to the throughput of MERFISH. EX2053, 11048 (“to reduce measurement time, we used multicolor imaging.”). Therefore, it is my opinion that a POSA would not have had a reason to consult Cai’s disclosures for any teaching of “two or more colors of light” to improve the throughput of Chen’s 2D MERFISH setup. Moreover, Chen’s acknowledgement of the limited multiplexing capabilities of smFISH and seqFISH would have further deterred a POSA away from considering the seqFISH teachings in Cai. *See* EX1003, 002 (“application of the smFISH approach ... remains limited by the number of RNA species that can be simultaneously measured in single cells”); *see also* EX2054, 16 (referring to smFISH and seqFISH as being “inadequate” and “not a practical approach to multiplexing.”); EX2055, 16-17. As a POSA would have understood the limitations of smFISH and seqFISH, a POSA interested in MERFISH protocols would have had no reason to consult Cai.

103. Third, Dr. Czymmek argues that Cai “teaches using seqFISH to

profile nucleic acid molecules and obtain ‘three-dimensional spatial information.’”

EX1002, ¶206 (citing EX1004, 21:50-51). Dr. Czymmek cites a single sentence in Cai that states “in some embodiments, an imaging step provides three-dimensional spatial information of a target.” EX1004, 21:50-51. The authors of Cai do not further elaborate upon this sentence, and the following sentence refers to a different embodiment entirely which “quantifies a target.” EX1004, 21:51-52. Dr. Czymmek’s only addition to this statement is his following sentence, where he argues that Cai teaches a z-axis motion control that would “permit three-dimensional interrogation through a stack of focal planes.” EX1002, ¶206.

However, Dr. Czymmek’s argument does not demonstrate what motivation a POSA would have to modify Chen’s 2D MERFISH setup to 3D MERFISH to arrive at the claimed volumetric imaging system. The cited disclosures from Cai merely reflect a POSA’s general knowledge in the art that methods of 3D imaging—such as z-stacking—did in fact exist. For example, my own publications available at the time highlighted this general knowledge of 3D fluorescence imaging techniques and the challenges associated with 3D imaging techniques. *See* EX1019, 003-005 (discussing “Super-resolution in 3D,” 3D imaging with “Structured Illumination Microscopy (SIM),” and “STORM & PALM in 3D.”); 001 (discussing that “imaging cells and subcellular structures in three-dimensional environments brings with it challenges”); 001 (noting the article’s objective is to

“discuss a few specific microscopy modalities that we believe will be particularly advantageous in imaging cells and subcellular structures in physiologically relevant 3D environments.”). However, the 554 patent claims *a complete imaging system* (including stage, fluidics system, optical system, z-axis control, and computing system) and not just the application of 3D imaging. The single generic sentence in Cai would not have given a POSA the motivation or rationale to modify Chen’s 2D MERFISH setup to arrive at the claimed system of the 554 patent. Rather, Cai’s single sentence is no different than citing the background knowledge in the art. *See e.g.*, EX1019, 001. At most, a POSA would have been motivated to apply smFISH HCR signal amplification method—disclosed in Cai and discussed in Section IX.E.1 above—to improve the SNR as taught by Cai. *See* EX1004, 44:34-40 (describing the HCR method wherein the “[a]mplification of FISH signals allows large FOV imaging of brain slices with 20x low NA objectives” to “increase the signal-to-background” of smFISH). Furthermore, Cai’s single sentence does not indicate how difficult of a feat it would be to modify Chen’s 2D MERFISH imaging setup into a volumetric imaging system for 3D imaging in view of Cai due to the lack of axial resolution, the problem of 3D photobleaching, and the poor SNR and SBR for 3D applications of Chen’s highly multiplexed MERFISH setup. Nor does Dr. Czymmek provide any explanation in this regard.

c. A POSA would have had no reason to modify Chen's highly multiplexed setup to arrive at 3D MERFISH

104. Beyond the lack of viable reasons given to modify Chen's imaging setup, it is my opinion that evidence in the art also shows that a POSA would not have had a reason to modify Chen's imaging setup to arrive at the claimed volumetric imaging system *while still satisfying MERFISH's goal of high multiplexing*. For one, even if a POSA were motivated to add more colors to Chen's 2D MERFISH protocol, this modification would have been for the purpose of further increasing multiplexing and throughput, not for the purpose of obtaining 3D positional information for thousands of RNA molecules. A POSA would have understood that Chen's objective in developing the MERFISH imaging setup was increasing multiplexing in two dimensions. *See supra* IX.D.1. A POSA would therefore be motivated, if at all, to further modify the MERFISH setup to increase the touted strength of the setup—highly multiplexed 2D imaging—rather than overhaul the setup to pursue a different kind of imaging entirely. This motivation to increase the multiplexity of 2D MERFISH imaging—rather than adopt MERFISH for 3D imaging—is reflected in later work from the same research group that developed MERFISH. EX2053, 11047. The authors of the study reported “an improved MERFISH method that drastically increases the throughput of this technique,” allowing the authors to “perform spatially resolved gene expression profiling of ~40,000 cultured human osteosarcoma (U-2 OS) cells in a

single 18-h experiment.” EX2053, 11047. The research group once again exclusively imaged their target cells in 2D. *See e.g.*, EX2053, Suppl. Materials, p. 3 (“A complete MERFISH measurement of ~ 500 FOVs covering 19.8 mm^2 required 12 h, and measurement of $\sim 1,000$ FOVs covering 40.8 mm^2 required 18 h.”). The continued focus on the throughput of MERFISH and the number of resolved transcripts confirms that the authors continued to place increased multiplexity as the most fundamental aspect of MERFISH.

105. A POSA would have understood that the greater multiplexity of Chen’s 2D MERFISH arrangement would lead to increased imaging challenges, especially for any attempts at 3D imaging. For example, Chen warns that “[a]s the degree of multiplexing is increased, it is important to consider the potential increase in the density of RNAs that need to be resolved in each round of imaging.” EX1003, 008. As explained above, a POSA would have known that as signal density in wide-field microscopy increases, the resolution, SNR, and SBR will decrease while the complexity of data analysis increases, a problem which would particularly impact axial resolution during attempts to image multiple planes through a thick sample. *See supra* Section VI.C. These issues with resolving individual nucleic acids would become even worse when attempting to image multiple planes in a sample for 3D imaging, as overlapping PSFs from large populations of fluorescently-labelled nucleic acids are more problematic in 3D

than 2D (due to the elongation of the PSF in the axial dimension) and a reduction in SNR and SBR from increasing numbers of out-of-focus molecules (whose background signal would make it harder to precisely localize molecules in each in-focus plane). *See supra* Section VI.C. Furthermore, a POSA would have known that Chen's use of wide-field imaging—even when used alongside oblique incidence excitation—would lower SNR and SBR by extinguishing fluorescent signals (photobleaching) outside of the focal plane in thick samples, causing a decrease in signal over time for attempted 3D MERFISH. The cumulative effect of signal density and photobleaching would ultimately render 3D imaging problematic, as it would be harder to localize single nucleic acids within the focal plane with sufficient precision (due to the lowering of SNR and SBR), and potentially impossible to accurately count and localize molecules outside of the focal plane (due to out-of-plane photobleaching in Chen's setup). The need to image and localize more fluorescently labeled RNA molecules in multiple focal planes, each with a high degree of multiplexing, would also be accompanied by an increased complexity of the procedure, which would further compound the already lengthy duration of the MERFISH protocol. *See Supra* Section VI.C; *see also* EX1003, 012 (describing how “[a]n entire [2D] experiment was typically completed in ~20 hours.”). A POSA would understand that for any attempts to modify Chen's 2D MERFISH setup into a volumetric imaging system for

determining three-dimensional positional information of nucleic acid molecules, a POSA would have to reduce multiplexity to improve resolution and limit complexity, thereby destroying the basic objective of MERFISH.

106. Dr. Czymmek asserts that a POSA “would have recognized that 3D single molecule approaches were well-known in the art.” EX1002, ¶207. In my opinion, Dr. Czymmek’s cited art for “3D FISH” does not provide a POSA with a reason to modify Chen in view of Cai, as these references either do not suggest 3D imaging or do not account for the impact of PSF, SNR, SBR, and signal density on axial resolution that would be expected during attempts to determine 3D positional information of highly multiplexed nucleic acid molecules. For example, Zessin disclosed 3D super resolution microscopy single-molecule localization of histone 2B and chromosomal DNA rather than 3D multiplexed imaging of small RNA molecules. EX1052, 002. Perillo only disclosed experiments imaging a single RNA species (“RunX2”) rather than the massive multiplexing found in Chen’s 2D MERFISH setup. EX1011, 003. Likewise, Larsson disclosed collecting z-stacks of images specifically to create maximum intensity projections without any form of localization data. EX1008, 002. Femino disclosed only basic 3D FISH for a single RNA species, rather than the massive multiplexing found in Chen’s 2D MERFISH setup. *See* EX1010, 017 (disclosing that probes were “targeted to the 3’-UTR of β -actin mRNA”). Brileya only uses two probes in its 3D FISH experiment targeted

toward whole biofilms, and does not appear to perform imaging on the level of single molecules or report any axial information for its study. EX1039, 003; *see also* EX1039, FIGs. 3 and 5 (showing 2D images of biofilms wherein FISH probes were used to visualize cocultured biofilm strains). Similarly, Tirichine performs 3D FISH on whole plant nuclei rather than individual nucleic acid molecules without massively multiplexed nucleic acids. EX1049, 004; *see also* EX1049 FIGs. 1-2. Finally, I understand Shah is not prior art to the 554 patent, as it was published in 2018. EX1020, 001.

107. Accordingly, it is my opinion that a POSA would not have had a reason to modify Chen's 2D MERFISH imaging setup to arrive at the claimed volumetric imaging system for 3D MERFISH, either in view of Cai or in view of the general knowledge of the art.

d. Dr. Czymmek does not explain what specific modifications the POSA would have made to Chen's setup to arrive at the claimed system

108. Even if a POSA were motivated to modify Chen's imaging setup in view of Cai and Shen, I note that Dr. Czymmek does not explain with any specificity what those modifications would have been or how a POSA would have made them to convert Chen's 2D MERFISH imaging setup into a volumetric imaging system for 3D MERFISH. Instead, Dr. Czymmek simply argues that Cai teaches a POSA to 1) add more fluorescent colors to increase multiplexity of

Chen's 2D MERFISH setup and 2) add a z-axis motion control system. EX1002, ¶172, ¶206. However, as discussed above, these modifications are already disclosed in Chen for 2D MERFISH, and a POSA would thus have had no reason to look to Cai for such teachings. Regardless of Cai, the POSA still would have performed MERFISH for 2D imaging because that is what Chen taught for its methods and imaging setup. The POSA would have known that adding more colors to Chen's imaging setup would not have changed the fundamental design of the arrangement for 2D imaging of multiplexed RNA molecules. *See* EX1003, 008 (disclosing that "using multiple colors" could help mitigate the downsides of increased multiplexing in the 2D MERFISH setup).

109. Moreover, as I discussed in detail above, it is my opinion that a POSA would *not* have been motivated to modify Chen's 2D MERFISH imaging setup into a system for volumetric imaging due to the increased complexity and difficulty 3D imaging would create in conjunction with MERFISH. As I outline above, the stated goal of Chen's 2D MERFISH setup was to increase multiplexing and throughput for simultaneous imaging of nucleic acids. Modifying Chen's 2D MERFISH imaging setup into a volumetric imaging system for 3D MERFISH would act against this objective by requiring a reduction in the ability to multiplex due to decreases in resolution and SNR and increases in data complexity and computing power that come with 3D imaging. Given this conflict in goals, it is my

opinion that a POSA would have no reason to modify Chen's 2D MERFISH imaging setup to arrive at the claimed 3D system of the 554 patent.

D. A POSA would have had no reasonable expectation of successfully modifying Chen's setup to arrive at the claimed system

110. Dr. Czymmek asserts that a POSA "would have recognized that 3D single molecule approaches were well-known in the art" and that "a POSA would have had a reasonable expectation of success in combining the references" to yield 3D MERFISH. EX1002, ¶¶207-208. As I explain below, I disagree with Dr. Czymmek because a POSA would understand that (i) adapting Chen's 2D MERFISH setup for 3D MERFISH in view of Cai would face significant issues with axial resolution—unaddressed by Dr. Czymmek, (ii) adapting a multiplexed method like Chen's 2D MERFISH for 3D imaging would create increased problems with computational complexity, and (iii) these same issues would apply to the other prior art that Dr. Czymmek cites to support a reasonable expectation of success in adapting Chen's multiplexed 2D MERFISH to 3D.

111. First, a POSA would have understood that axial resolution and aspects of fluorescence microscopy would create challenges when attempting to adapt Chen's 2D MERFISH setup for 3D imaging. For one, it was known in the art that multiplexing can create challenges for fluorescence microscopy by greatly increasing signal density. For example, Chen warns that "[a]s the degree of multiplexing is increased, it is important to consider the potential increase in the

density of RNAs that need to be resolved in each round of imaging.” EX1003, 008; *see also* EX2051, 3 (noting that “[w]hen the density of transcripts is high, diffraction-limited fluorescent spots of the same color will overlap and make barcode readout impossible.”). This would be particularly noticeable during attempts to acquire 3D images of thick samples, wherein axially elongated PSFs from adjacent focal planes in a densely-packed, multiplexed sample would overlap and make it increasingly difficult to resolve individual molecules. *See* EX1019, 001 (discussing the “Z-stretch” in fluorescence microscopy and its impact on resolution). Furthermore, a POSA would have expected the SNR and SBR to become a challenge when attempting to convert Chen’s highly multiplexed 2D MERFISH setup to 3D in view of Cai. For example, Chen’s 2D MERFISH setup used wide-field imaging, which is known to have poor axial resolution and to illuminate planes above and below the focal plane. EX2046, 920; EX1012, 004. A POSA would have understood that, when applied to 3D fluorescence imaging, a highly multiplexed technique like Chen’s 2D MERFISH would face a low SNR due to 1) significant background from fluorescence from out-of-focus planes, and 2) decreased signal due to photobleaching of fluorescent probes by illumination outside the focal plane. Therefore, a POSA would not have had a reasonable expectation of success because of the lack of axial resolution, the problem of 3D photobleaching, and poor SNR and SBR for 3D applications in Chen’s highly

multiplexed MERFISH setup designed for 2D applications in thin cells, all of which would be exacerbated in an attempt at modification for 3D imaging in view of Cai.

112. Second, a POSA would also have expected the signal density of a 3D multiplexed sample to create challenges with computational complexity. Cai itself notes that “[b]ecause smFISH often uses two or more colors for a target, it produces high density of objects in the image” that “can increase the complexity of data analysis.” EX1004, 1:67-2:3 Furthermore, this high density of objects is particularly problematic for multiplexing and barcoding schemes, where scaling a technique to high degrees of multiplexing can necessitate more difficult, complex, and time-intensive computations during image processing. *See e.g.* EX2051, 7 (discussing that scaling a spatial barcoding technique “beyond 1,000 genes” required a “more sophisticated computational algorithm.”). A POSA would have understood that this computational challenge would be further exaggerated if attempting to image multiple planes through a sample for 3D imaging, as thousands of RNA molecules imaged in one 2D plane would now then be multiplied to numerous planes throughout the depth of the sample. Therefore, it is my opinion that this computational complexity would diminish the expectation of success in adapting Chen’s highly multiplexed 2D MERFISH method to 3D imaging.

113. Third, challenges in adapting Chen's 2D MERFISH imaging setup for 3D imaging would also diminish the impact of Dr. Czymmek's other prior art citations for reasonable expectation of success. Dr. Czymmek cites multiple sources to purportedly show that a POSA would have had a reasonable expectation of success in combining Chen with Cai. *See* EX1002, ¶207 (citing EX1052 to show that a POSA "would have recognized that 3D single molecule approaches were well-known in the art."), ¶208 (citing EX1010, EX1008, and EX1011), ¶294 (citing EX1039), ¶288 (citing EX1049), ¶59 (citing 1020). However, as I discussed above, none of these sources capture the additional complexities of 3D fluorescence imaging introduced by RNA multiplexing. For example, Zessin performed 3D super resolution microscopy single-molecule localization of histone 2B and chromosomal DNA rather than multiplexed imaging of hundreds or thousands of small RNA molecules. EX1052, 002. Similarly, Perillo outlined experiments with imaging of a single RNA species rather than the multiplexing 100s to 1000s of RNA species found in Chen's 2D MERFISH setup, and Larsson likewise does not assign any form of axial localization in its data and only collected images as a z-stack to create a maximum intensity projection. EX1008, 002; EX1011, 003. Femino disclosed only basic 3D FISH for a single RNA species, not massive multiplexing RNAs like Chen's MERFISH. *See* EX1010, 017 (disclosing that probes were "targeted to the 3'-UTR of β -actin mRNA."). Dr.

Czymmek also asserts that Brileya discloses “3D-FISH ... to determine colocalization patterns of various biomarkers” and the use of “a water dipping objective to perform confocal laser scanning microscopy in 3D-FISH to investigate biofilms.” EX1002, ¶103, ¶294. However, Brileya only uses two probes in its FISH experiment (one for each biofilm coculture), and does not appear to perform imaging on the level of single molecules or report any axial information for its study. EX1039, 003; *see also* EX1039, FIGs. 3 and 5 (showing 2D images of biofilms wherein FISH probes were used to visualize cocultured biofilm strains). Furthermore, Brileya’s setup used a low NA (“63x 0.9 NA”) water objective lens that a POSA would have understood to be unsuitable for high-resolution 3D imaging of highly multiplexed single molecule samples, as imaging such samples—especially in 3D—would ideally demand high resolution and high signal collection. *See supra* [IX.E.1.] (discussing how Chen’s high NA setup would have resolution more suited to imaging high density signals). Similarly, Tirichine does not report the 3D location of FISH puncta. Instead, the authors only took 3D images of whole nuclei, and the data presented are either maximum intensity projections from 3D data or 2D images of cryosections. EX1049, 004; *see also* EX1049, FIGs. 1-2 (showing maximum intensity projection data and cryosection images). Finally, Shah published in 2018 and thus I understand it is not prior art to the 554 patent. EX1020, 001. In my opinion, the above-cited prior art does not

establish a reasonable expectation of success in modifying Chen's 2D MERFISH setup for 3D imaging, as the art and Dr. Czymmek do not show 3D imaging with high multiplexing or provide any indication as to how to mitigate issues of signal density, SNR, and computational complexity that would result from such a modification.

114. As discussed above, a POSA would have understood that modifying Chen's highly multiplexed 2D MERFISH setup would create significant challenges to any attempt at modification for 3D imaging. Dr. Czymmek's cited prior art for "3D single molecule approaches" does not discuss the additional 3D imaging challenges that would need to be addressed to achieve 3D imaging with Chen's 2D MERFISH setup. Therefore, it is my opinion that Dr. Cymmek's cited art does not establish that there would be a reasonable expectation of success for a POSA seeking to modify Chen's 2D MERFISH setup for 3D imaging.

E. Dependent claims 37-70

115. I understand that a dependent claim requires all of the limitations of the claim from which it depends. Claims 37-70 each depend directly or indirectly from claim 36. EX1001, 34:18-36:26. Therefore, I understand that claims 37-70 require all of the limitations of the volumetric imaging system of claim 36. Thus, the deficiencies in Dr. Czymmek's obviousness opinions for claim 36 also apply to dependent claims 37-70.

F. Objective Indicia further confirm the nonobviousness of the claimed system.

116. I understand that when considering the obviousness of an invention, one should also consider whether there is any objective evidence that supports the non-obviousness of the invention. I understand that objective indicia include of non-obviousness includes such factors as contemporaneous responses to the inventions, commercial success, long-felt need, failure of others, industry-wide praise, and unexpected results. Dr. Czymmek failed to address objective indicia of nonobviousness in his declaration.

117. As I discuss below, it is my opinion that the innovative nature of the 554 patent's claimed system is evident in the industry praise of 10x's Xenium and Bruker's/NanoString's CosMx imaging systems. Furthermore, it is my opinion that the volumetric imaging system of the 554 patent embodied in these products satisfied a long-felt, but unmet need for a complete volumetric imaging system for determining 3D positional information of one or more nucleic acids.

1. Xenium and CosMx are commercial embodiments of the 554 patent's claimed volumetric imaging system

118. I understand that for objective evidence to have weight there must be a nexus between the evidence and the claim as a whole. I understand that a patentee is entitled to a presumption of nexus between the objective indicia evidence and the patent claim if the evidence is tied to a specific product that

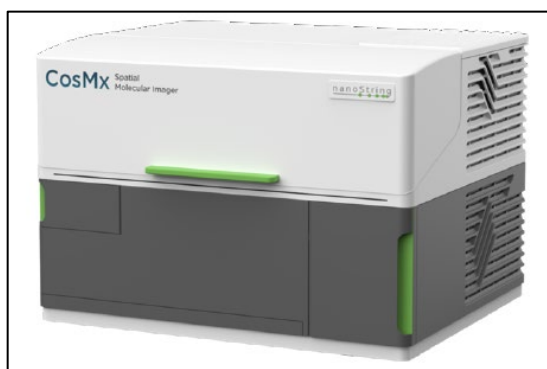
embodies the claimed features and is coextensive with them. I also understand that even if a commercial embodiment is not coextensive with claimed features in a patent, then objective indicia will still have a nexus if the objective indicia evidence is the direct result of the claimed invention's unique characteristics.

a. Claim 36 (preamble): A system comprising:

119. In my opinion, Xenium and CosMx have a nexus with the claims of the 554 patent because both imaging systems are commercial embodiments of the 554 patent's claimed system. Both Xenium and CosMx (each shown in the figures below) are complete, single-unit volumetric imaging systems for determining three-dimensional positional information of nucleic acid molecules.



10x's Xenium



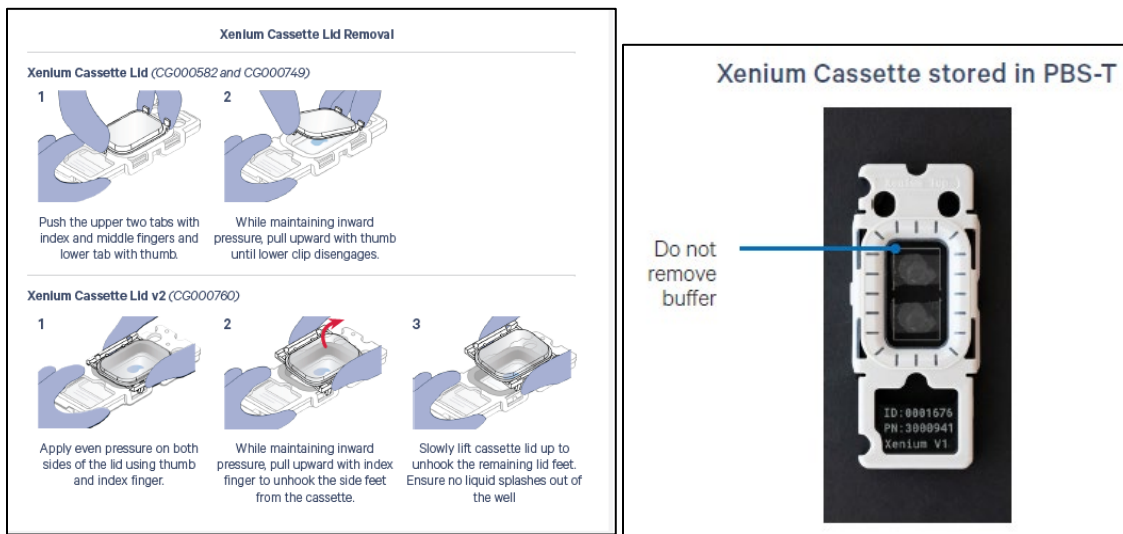
Bruker's/NanoString's CosMx

EX2060, 1; EX2061, 9. As I discuss below, these complete volumetric imaging systems are coextensive with the claimed volumetric imaging system in the 554 patent.

b. Claim 36[a]: (a) stage comprising: (i) a sample holder configured to receive a substrate comprising a sample having a volume; (ii) a temperature control apparatus

operatively coupled to said sample holder; and (iii) an XY Motion control system;

120. The system of claim 36 comprises a “sample holder configured to receive a substrate comprising a sample having a volume.” EX1001, 33:31-32. In line with the claim, the Xenium platform utilizes a cassette carrier, which is configured to receive a cassette containing a tissue sample. EX2060, 17, 29, 70. The cassette in the cassette carrier constitutes the substrate in the claim, while the tissue sample constitutes the sample having a volume. This cassette carrier setup is demonstrated in the figures below, taken from the Xenium User Guide:



EX2060, 29, 70. In the Xenium User Guide, the cassette carrier is shown holding the cassette, upon which a sample is loaded (left panel). The User Guide notes that “[c]assettes should always be stored *hydrated* with the recommended reagent and stored at the recommended temperature *to maintain sample integrity*.” EX2060, 92. The manufacturer’s instructions regarding the cassettes indicate that 1) the

cassettes are directly associated with a sample and 2) that the tissue sample has a volume that must be maintained through hydration. Therefore, the combined cassette and tissue sample constitute a “substrate comprising a sample having a volume,” whereas the cassette carrier is configured to receive that substrate as demonstrated in the left panel above. CosMx likewise utilizes flow cells with slides configured to receive a substrate comprising a sample having a volume (in this case, a tissue sample). *See* EX2061, 10 (describing a CosMx workflow overview wherein the user loads slides with tissue samples). In the CosMx Slide Preparation Manual, the flow cell is said to “enable[] input of a tissue section sample into the CosMx SMI instrument for spatial profiling.” EX2062, 132. The manual states that the flow cell “affixes to a 3 inch x 1 inch standard pathology grade slide with mounted tissue, creating an imageable fluidic channel.” EX2062, 132. These instructions therefore indicate that the flow cell is a sample holder configured to receive the combined slides and tissue samples. Likewise, the slide and tissue sample represent a substrate (the slide) and a sample having a volume (the tissue sample).

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

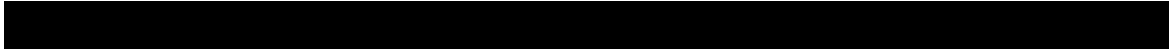
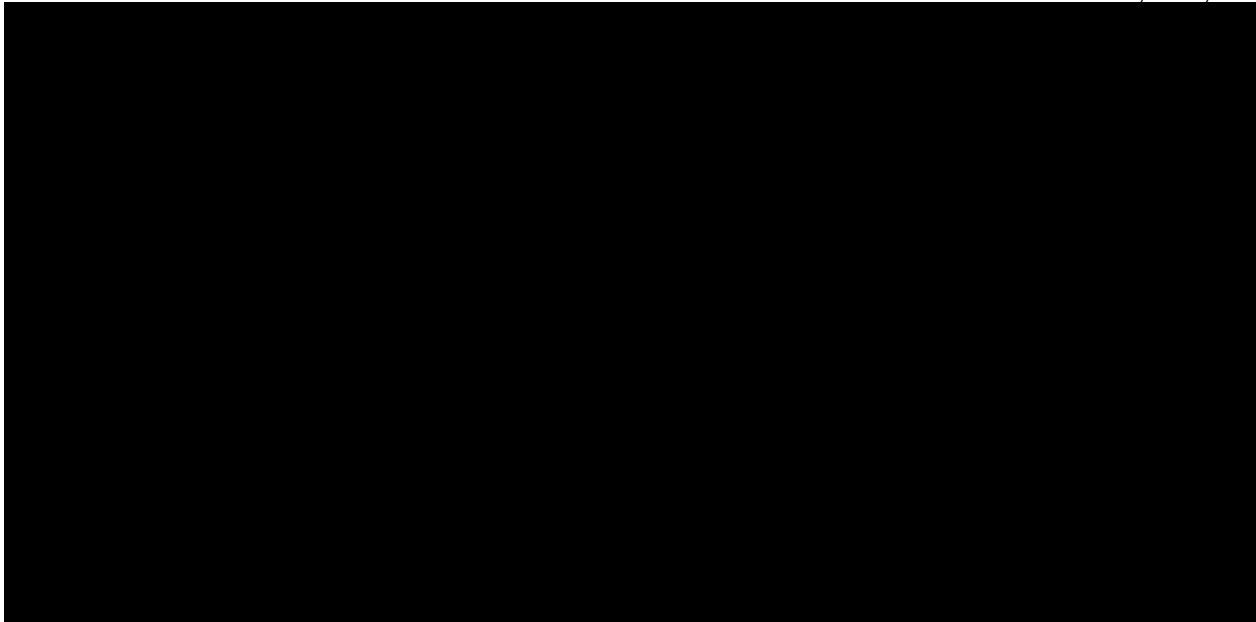
[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

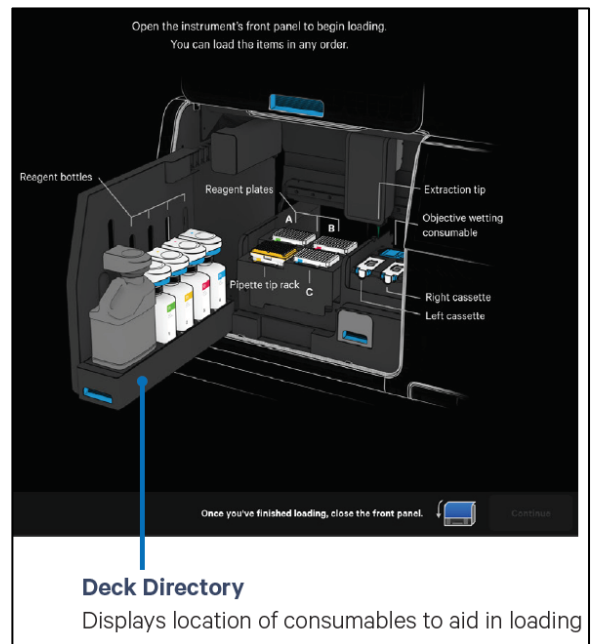
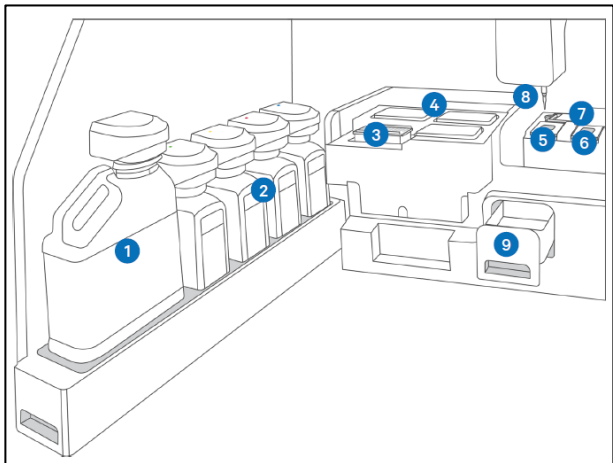
[REDACTED]



- c. Claim 36(b): a fluidics system comprising: (i) one or more reservoirs configured to contain one or more fluid reagents; and (ii) a fluid dispenser operationally coupled to a pump, which pump is configured to provide said one or more fluid reagents from said one or more reservoirs to said sample holder; and**

123. The system described in claim 36 also comprises a “fluidics system” comprising “one or more reservoirs” and a “fluid dispenser.” EX1001, 33:35-42. As shown in the figure below, the Xenium system likewise contains these elements. The Xenium system contains “one or more reservoirs” in the form of reagent bottles (“2” in the figure below) stacked along a Deck Directory as well as

reagent plates (“4” in the figure below). EX2060, 18, 66. Likewise, Xenium contains a “fluid dispenser” in the form of a pipette using pipette tips stored in a rack. *See* EX2060, 67 (providing instructions for loading the “Pipette Tip Rack.” The pipette tip rack is shown as a “3” in the left panel of the figure below. Each of these components is part of an overall “fluidics system” as further illustrated by the Xenium guide’s instructions referring to cleaning the “fluidics system.” EX2060, 77, 85. Thus, Xenium contains a “fluidics system” comprising “one or more reservoirs” (reagent bottles in the Deck Directory) and a “fluid dispenser” (the extraction tip). Similarly, CosMx also comprises a fluidics system with “bulk reagent bottles” that must be “loaded before starting a run,” and are placed in color-coded reservoirs in the instrument. EX2061, 12, 51-54.



EX2060, 18, 66.

- d. **Claim 36(c): an optical system comprising: (i) an objective; (ii) two or more light sources, wherein each light source of said two or more light sources is configured to deliver light to said volume, wherein said two or more light sources are configured to emit two or more colors of light; and (iii) one or more detectors configured to receive light signals from said volume;**

124. Claim 36 also discloses an “optical system” for the claimed invention, comprising “two or more light sources” and “one or more detectors.” EX1001, 33:43-54. These elements are once again reflected in the User Guide for the Xenium system. The guide discloses that the Xenium system “captures vertical stacks of images at every cycle (of fluorescent probe hybridization, imaging, and probe removal) and in every channel for multiple fields of view,” further adding that the process begins with a “sample scan.” EX2060, 26, 72. This indicates the need for a z-axis control system to capture the vertical stacks and at least one detector to capture the images. The User Guide also includes extensive instructions for image scanning that repeatedly refer to adjusting or turning multiple channels on or off. EX2060, 73. The multiple channels of the Xenium system indicate that multiple sources of light are used in the system, for which at least one detector would be needed to detect said light channels. Likewise, CosMx includes a range of excitation and emission spectra that are used during “scanning” of the flowcells for image acquisition. EX2061, 14, 48-49, 55-57. Like the Xenium User Guide, the CosMx User Manual includes information on the multiple channels used in the

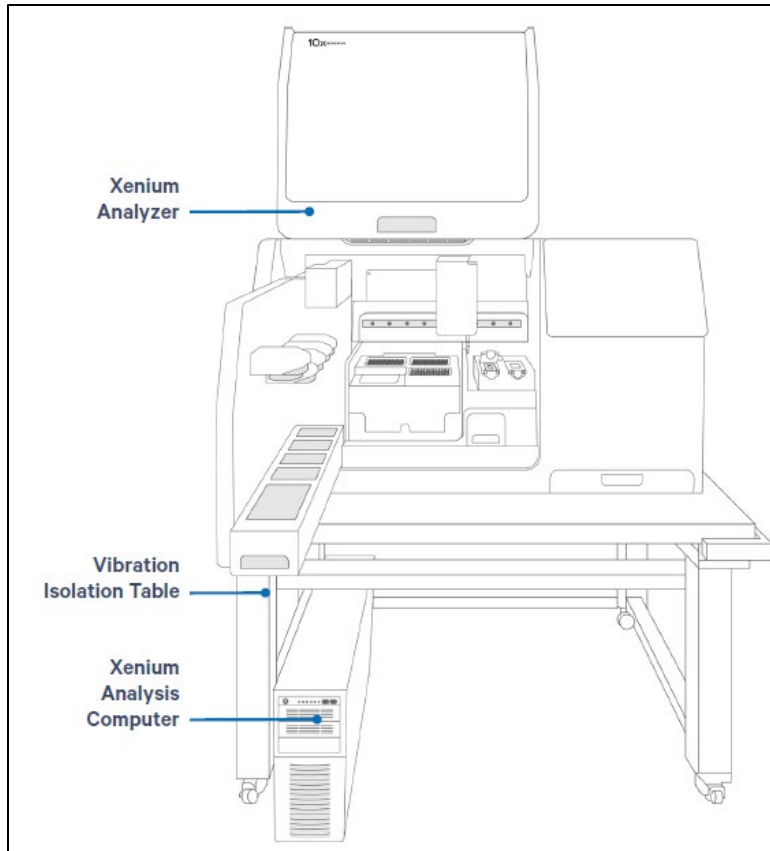
system. EX2061, 58.

- e. **Claim 36(d): a Z-axis motion control system configured for Z-motion of an objective focal plane through said volume; and**

125. Claim 36 also discloses a Z-axis motion control system configured for Z-motion of an objective focal plane. Xenium system's ability to "capture[] vertical stacks of images" reflects the presence of a z-control system in order to obtain said z-stacks. EX2060, 26. Likewise, CosMx "provides a high-resolution subcellular image-based readout by identifying the X, Y, and Z coordinates of each target molecule." EX2065, 3.

- f. **Claim 36[e]: a computing system comprising at least one computer readable storage medium having program instructions stored thereon, which program instructions are executable by at least one processor of said computing system to cause said at least one processor to perform a method comprising : receiving a plurality of images of at least a portion of said volume from a plurality of read-out cycles, wherein said plurality of images comprises a plurality of light signals received from said volume during said plurality of readout cycles, wherein said plurality of light signals is associated with one or more nucleic acid molecules within said volume, wherein each light signal of said plurality of light signals has a three-dimensional spatial position within said volume; determining a plurality of optically-encoded signals from said plurality of light signals obtained from said plurality of read-out cycles, wherein each optically-encoded signal is associated with a nucleic acid molecule of said one or more nucleic acid molecules, wherein said plurality of optically-encoded signals comprises said two or more colors of light detected by said one or more detectors in series; identifying said one or more nucleic acid molecules based on said plurality of optically- encoded signals; and determining three-dimensional positional information of said one or more nucleic acid molecules.**

126. The system in claim 36 also comprises a “computing system” for “determining three-dimensional positional information of [] one or more nucleic acid molecules.” EX1001, 33:55-17. The Xenium system likewise comprises a “Xenium Analysis Computer” depicted in the figure below from the User Guide:



EX2060, 16.

127. The User Guide includes disclosures that the Xenium Analysis Computer’s software analyzes multiple fields of view and subsequently “processe[s], correct[s] and stitche[s] to build a single seamless image representing the tissue section.” EX2060, 26. The Guide also adds that “[t]he pipeline detects puncta in every cycle and every image in order to observe all potential mRNA. These puncta are decoded into gene IDs, and each decoded transcript is assigned a quality score.” EX2060, 26. The described process of “build[ing] a single seamless image representing the tissue section” demonstrates the ability to use the Xenium computing system to 3D reconstruction of tissue samples. EX2060, 26. Likewise,

the ability to detect puncta to “observe all potential mRNA” and decode them demonstrates computing exact positional information for mRNA. EX2060, 26. Similarly, CosMx also comprises a computing system for determining three-dimensional positional information of nucleic acid molecules. *See e.g.*, EX2065, 3 (disclosing that “CosMx SMI provides a high-resolution subcellular image-based readout by identifying the X, Y, and Z coordinates of each target molecule, which is translated to spatial location data and exported to the cloud”), *see also* EX2061, 55 (“FOVs are selected and saved, the CosMx SMI instrument begins Reporter Cycling and Data Acquisition.”).

128. Accordingly, the claims of the 554 patent are embodied in the Xenium and CosMx imaging systems, as these systems embody the claimed features of the volumetric imaging system of claim 36 and are coextensive with them.

2. The volumetric imaging system of the 554 patent satisfied a long-felt, but unmet need for a complete volumetric imaging system

129. I understand that evidence of a long-felt, but unmet, need in the art can weigh towards non-obviousness of an invention. I further understand that a showing of long-felt need requires (i) that the need was recognized by a POSA; (ii) the need was not satisfied by another before the invention; and (iii) the invention satisfied the long-felt need. In my opinion, the rapid adoption of the Xenium and CosMx imaging systems demonstrates a long-felt need in the art for a system for

volumetric imaging. Prior to the commercial availability of Xenium and CosMx, few labs would have had access to a complete system for volumetric imaging. At best, labs would have had access to home-built setups that were unwieldy, sprawling, and laborious to assemble or difficult to maintain. *See supra* Section VI.A. I understand that both Xenium and CosMx became commercially available in late 2022. I further understand that Xenium’s worldwide sales between Q2 2022 and Q4 2024, following Xenium’s release, were approximately \$127.8 million. Additionally, I understand from EX2040 that more than 250 Xenium Instruments were sold in the first year since launching with researchers validating Xenium’s sensitivity, specificity, and high throughput. EX2040, 5, 8. In my opinion, this rapid growth in sales was the direct result of essential aspects of the 554 patent—namely, the complete volumetric imaging systems’ capability to provide “high resolution spatial data” while “streamlin[ing] the process from tissue handling to data interpretation.” EX2072, 13; EX2071, 2. The combined surge in purchases and recognition in the scientific community (discussed below) help to show an existing need for a complete volumetric imaging system prior to the release of Xenium and CosMx. Furthermore, without the benefit of the technology of the 554 patent, Xenium and CosMx would not have been a ready-to-use volumetric imaging system capable of generating high-resolution spatial data and would not have experienced the same degree of success. For example, without the technology

of the 554 patent Xenium and CosMx would not possess the integrated hardware and software required for effective in situ workflow in a volumetric imaging system. *See e.g.* Section VII.A.

3. Those in the industry praised Xenium’s streamlined workflow and ability to provide high-resolution spatial data

130. I understand that praise in the industry for a patented invention can be evidence that the invention is non-obvious. In my opinion, there is strong evidence of industry praise and recognition for 10x’s Xenium imaging system that is directly linked to the features in claim 36 of the 554 patent. Publications utilizing Xenium have praised the platform for the breadth of the system’s sequencing ability, the smooth workflow provided by the integrated system, and the ability of the platform to provide high-resolution spatial data for nucleic acid molecules. For example, Dr. Jeremie Oliver Piña of the NIH recently lauded Xenium’s ability to provide “*greater depth and breadth* than prior studies could....” EX2070, 2. Dr. Piña further praised the Xenium system’s innovative combination of hardware and software, noting that “[t]he intuitive design of [Xenium’s] *bench workflow and post-run analysis software* may be the most valuable aspect of this high-throughput in situ technology to accelerate science. EX2070, 2. Similarly, Dr. Luciano Martelotta of the University of Adelaide lauded Xenium for being “a cut above the rest” because it “*streamlines the process from tissue handling to data interpretation*, making it faster and easier to get insights into biology.” EX2071, 2.

Likewise, Peroni et al. praised Xenium for the platform's ability to accurately determine the three-dimensional positional information of nucleic acid molecules. In particular, Peroni et al. lauded Xenium's "ability to provide *high-resolution spatial data*," adding that the ability "makes Xenium *an invaluable tool* for advancing precision medicine." EX2072, 13. Dr. Simon Gregory of Duke University similarly praised Xenium for "providing [his team] with unprecedented insight into the molecular pathology of disease at an *incredible resolution*." EX2071, 1.

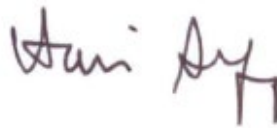
131. Accordingly, the Xenium platform has been praised for the breadth of the system's sequencing ability, the smooth workflow of the integrated imaging system, and its computing system capable of streamlining the data interpretation capability and ability to provide high resolution spatial data for nucleic acid molecules.

X. CONCLUSION

132. In signing this declaration, I recognize that the declaration will be filed as evidence in an *inter partes* review before the Patent Trial and Appeal Board of the United States Patent and Trademark Office. I also recognize that I may be subject to cross-examination in the case and that cross-examination will take place within the United States. If cross-examination is required, I will appear for cross-examination within the United States during the time allotted.

133. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Executed on this 10th day of December 2024.



Hari Shroff, Ph.D.