

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

NANOSTRING TECHNOLOGIES, INC.
Petitioner,

v.

PRESIDENT AND FELLOWS OF HARVARD COLLEGE,
Patent Owner

U.S. PATENT NO. 11,542,554

**DECLARATION OF KIRK CYZMMEK, PH.D. IN SUPPORT OF
PETITIONER FOR *INTER PARTES* REVIEW**

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I. INTRODUCTION AND ENGAGEMENT

I, Kirk Czymmek, declare as follows:

A. Introduction

1. I am over the age of twenty-one (21) and am competent to make this Declaration. I reside at 603 Martin Pointe Ct, Wildwood, MO 63011.

B. Engagement

2. I have been retained by counsel for NanoString Technologies, Inc. (“NanoString”) in the above-captioned *inter parte* review (IPR) matter as an independent technical expert to review and evaluate specific claims of the U.S. Patent 11,542,554 (“the ’554 Patent”). I have been asked to provide my opinion regarding whether a person of ordinary skill in the art (“POSA”) in the subject matter of the claims would find them anticipated or obvious over certain publications. My opinions are based on my skills, knowledge, training, education, and experience in matters of this nature, and my examination of the materials used in preparing this report. I expect to testify regarding the matters set forth in this declaration if asked to do so.

3. I am being compensated on an hourly basis for my work performed in connection with this matter. I have received no additional compensation for my work in this matter, and my compensation does not depend upon the contents of this report, any testimony I may provide, or the ultimate outcome of the proceeding.

II. BACKGROUND AND QUALIFICATIONS

4. I received my Bachelor of Science degree summa cum laude in Biological Sciences from the State University of Oswego, in Oswego, NY, in 1989. My Bachelor degree involved optical microscopy, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) as an integral part of my senior honors thesis. I began my Ph.D. work in the Department of Botany and Plant Pathology at Michigan State University in East Lansing, MI in 1989, which was completed in 1993. At Michigan State University, as part of the Center for Electron Optics, I was exposed to a broad array of imaging technologies including TEM, SEM, laser scanning confocal microscopy (LSCM) and atomic force microscopy (AFM), cryogenic preparative techniques including plunge-freezing, propane-jet freezing, high-pressure freezing, freeze-substitution and cryo-SEM. This included any photographic, analog and digital image acquisition, processing and analysis workflows.

5. Furthermore, I participated in research and instruction of students using an array of fluorescent probes on one of the first commercial LSCM (Zeiss LSM 010). I was a Teaching Assistant in the following courses (Plant Physiology, Fundamentals of Botany, Scanning Electron Microscopy, Transmission Electron and Laser Scanning Confocal Microscopy) and these courses focused on cell and plant biology or theory, application and included operation of confocal and electron

microscopes. My postdoctoral training began in 1993 at The DuPont Company in Central Research and Development, Plant Molecular Genetics Group in Wilmington, DE in the laboratory of Richard Howard, who has since retired from his position as a Research Fellow and cell biologist.

6. I was recruited by DuPont specifically for my broad microscopy skills including confocal, fluorescence probes and cryo-techniques expertise. As a postdoctoral scientist in Dr. Howard's laboratory, I developed improved methods for visualizing fluorescence labeling of proteins (including the use of genetically encoded green fluorescent proteins and reef coral fluorescent proteins), polysaccharides and nucleic acids in fungi and plants using cryo-techniques with light and electron microscopy. After my postdoc I joined the Confocal Business Unit at Noran Instruments, Inc. in Madison, WI as an applications scientist. In this role I worked closely with engineers to develop and debug our high-speed and 3D confocal microscope platforms (up to 480 frames/sec) and provided training for support sales and customers. This included the specification and extensive experience with fluorescent excitation and emission filters, training user on our acquisition, analysis and visualization software (including 3D deconvolution) and troubleshooting for a broad array of biological and materials applications.

7. In 1996, I joined the University of Delaware as the Laboratory Coordinator of the Electron Microscopy Facility which involved oversight, training

and sample preparation for two transmission electron microscopes and a scanning electron microscope and shortly thereafter added a LSCM. In 2001, I was promoted to a secondary appointment in the Department of Biological Sciences as an Associate Professor and the following year to the Directorship with the task to build a new Bio-Imaging Center in the newly built Delaware Biotechnology Institute which is part of the University of Delaware. The Delaware Biotechnology Institute is a state, industry and university partnership and I worked closely with architects to design a ~3000sq/ft internationally recognized state-of-the-art bio-imaging microscopy capacity by integrating a broad array of 2D and 3D microscopy platforms (i.e., AFM, SEM, TEM, LM, LSCM, super-resolution microscopy, laser capture micro-dissection with all supporting specimen preparation techniques and a host of advanced image processing software and hardware platforms to archive, process, analyze and visualize or digitalize datasets. As its founding director, my vision for the UD Bio-Imaging Center was to specialize in advanced microscopy tools with special emphasis on biological and soft materials in the life sciences, engineering, and agricultural sciences. In 2009, I was promoted to a primary Associate Professor position and, in 2010, I also received an Associate Professor appointment (secondary) in the Department of Plant & Soil Sciences. In 2012, I was appointed as a Senior Research Scientist in the Christiana Care Hospital System as part of the Helen F. Graham Cancer Center. During my 16 year tenure at the

University I was a member of at least 40 thesis committees primarily involving microscopy in the life sciences, engineering, and agricultural sciences.

8. I have served as an ad-hoc reviewer for The Cell Structure and Function and Shared Instrumentation Grant Study Sections for the National Institutes of Health, The United States Department of Agriculture and the National Science Foundation. I also review periodically for a number of journals including Langmuir, PLoS ONE, Plant Physiology, Plant Cell, Fungal Genetics and Biology, Protoplasma, iScience, Molecular Plant Microbe Interactions, Frontiers in Plant Science, Current Opinion in Plant Science and Plant and Soil. As an Associate Professor in the Department of Biological Sciences with funded research on fungal cell biology and plant pathogenesis, I received funding from the National Institutes of Health, the National Science Foundation and the United States Department of Agriculture. I was a member of the University of Delaware Research Council, The University of Delaware Core Facilities Task Force, the College of Arts and Sciences Strategic Planning Committee, the Center for Translational Cancer Research Steering Committee, the University of Delaware Core Facilities Task Force, The Provost's Magnetic Resonance Imaging Task Force, the Departmental of Biological Sciences Research Committee, the Department of Biological Sciences Alumni Relations Committee, part of the management team and serve as Director of Core Facilities for the NIH funded Delaware IDeA Networks for Biomedical Research

Excellence grant and Program Coordinator of Core Facilities for the NIH funded Centers for Biomedical Research Excellence grant.

9. In 2012, I joined ZEISS to build a world-class application, demonstration, and training center for the ZEISS microscopy portfolio in North America. ZEISS, headquartered in Germany, is a leading international technology company in the optics and optoelectronics industries which develops microscopy solutions for industrial metrology and quality assurance, the life sciences, materials research, and medical technology. There I was quickly promoted to Global Coordinator of applications, demonstration and training centers and then subsequently promoted to the role of Vice President of Global ZEISS Microscopy Customer Centers reporting to the Global Head of ZEISS Microscopy Sales and Service. This involved oversight of all global demonstration inventory and (8) customer centers world-wide (New York, NY & Pleasanton, CA USA, Cambridge, UK, Munich, DE, Oberkochen, DE, Tokyo, Japan, Singapore, Shanghai and Beijing, China). In this role, I had 23 direct reports with a total of 63 global Zeiss applications scientists. I regularly interfaced with, and provided considerable application and technical knowledge to, our academic and industrial materials and life science customers as well as internal sales, service, marketing, and research and product development teams on the application and development of our light, electron and x-ray products. I was on the ZEISS North America leadership team and the

ZEISS global Management Level 3 team, which consisted of the heads of each country, global region and Germany headquarters management.

10. In 2019, I joined the Donald Danforth Plant Science Center as a Principal Investigator and Director the Advanced Bioimaging Laboratory to build a capacity of cutting-edge advanced microscopy tools in plant sciences dedicated to producing more nutritious food and improving the environment for the benefit of humankind. With over 30 years of advanced microscopy experience, I have expertise in most forms of light, x-ray, 3D scanning and transmission electron microscopy, volume electron microscopy, atomic force microscopy, optical, confocal, multi-photon, single molecule imaging, structured illumination and single molecule localization super-resolution microscopy, deconvolution, Fluorescence Recovery After Photobleaching (FRAP), Förster Resonance Energy Transfer (FRET), multiplex microscopy, cryo-techniques, correlative microscopy and a host of image processing, analysis and visualization tools, including the application of artificial intelligence (machine and deep learning). I have over 120 refereed publications as this work has focused on developing and applying cutting-edge microscopy tools for imaging cells, tissues and biomaterials. I am currently on the executive council for the Microscopy Society of America as a Biological Sciences Director and am funded by the National Science Foundation, the Department of Energy, National Institutes of Health, the United States Department of Agriculture,

the Chan Zuckerberg Initiative, and private funding by Invaio Sciences. In June 2022, I co-founded the company Peptyde Bio, a company that discovers, designs, and characterizes novel peptides for use in agriculture. I served as its Chief Technology Officer and led a team of 9 scientists until it was sold in December 2023 to Invaio Sciences, a Flagship Pioneering company. I continue to lead this team as the principal investigator and consultant as part of a Strategic Research Agreement with the Danforth Center and my lab for a transition period of 18 months.

11. As part of my education and professional training and experience, I have used a broad array of microscopy technologies to understand various aspects of the structure, function and dynamics of cells and/or materials. While my primary funded research involves understanding how fungi and microbes grow and cause disease in plants, my professional positions by necessity resulted in the application of basic and advanced microscopy in its many forms and to many disciplines. A copy of my Curriculum Vitae, attached as Exhibit 1065, contains further details on my education, experience, publications, and other qualifications to render an expert opinion in this matter.

III. SUMMARY OF OPINIONS

12. In forming my opinions, I have relied upon my education, knowledge and the contributions to knowledge that I have made in the general areas of microscopy and bio-imaging. The specific areas that are most relevant include:

state-of-the-art bio-imaging microscopy that integrates various manual and automated capable microscopy platforms, i.e., AFM, SEM, TEM, LM, LSCM, super-resolution microscopy, and laser capture micro-dissection with all supporting specimen preparation techniques, with a host of advanced image processing software and hardware platforms to archive, process, analyze and visualize or digitalize datasets. I also have relied upon my education, knowledge and experience with most forms of light, x-ray, 3D scanning and transmission electron microscopy, atomic force microscopy, optical, confocal, multi-photon, single molecule imaging, structured illumination and single molecule localization super-resolution microscopy, deconvolution, expansion microscopy, Fluorescence Recovery After Photobleaching (FRAP), Förster Resonance Energy Transfer (FRET), multiplex microscopy, cryo-techniques, correlative microscopy and a host of image processing, analysis and visualization tools, including the application of artificial intelligence (machine and deep learning).

13. For the above mentioned platforms and microscopy approaches, my expertise and skills extend to any required routine and advanced sample preparation techniques and workflows such as, but not limited to, live cell imaging, vital stains and affinity probe labeling, chemical and cryo fixation, immunolocalization, *in situ* hybridization, hybridization chain reaction, cryo-, vibratome- paraffin- and acrylic/epoxy resin-based embedding and sectioning, to name a few.

14. For this work, I reviewed and considered all of the materials cited and discussed in this declaration.

15. As I explain in this declaration, it is my opinion that claims 36-70 of the '554 Patent are unpatentable for obviousness on the following grounds:

- **Ground 1:** Claims 36-39, 43, 44, 46, 47, 54, 56, 59, 60, and 63-69 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032);
- **Ground 2:** Claim 40 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032), in further view of Xiao (EX1027) and Ehrlicher (EX1028);
- **Ground 3:** Claims 41 and 70 over the combination of Zhuang (EX1003), Cai ((EX1004), and Shen (EX1032), in further view of Tirichine (EX1049);
- **Ground 4:** Claims 42 and 70 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032), in further view of Brileya (EX1039);
- **Ground 5:** Claim 45 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032), in further view of Fang-Yen (EX1029);

- **Ground 6:** Claim 48 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032), in further view of Richard (EX1030);
- **Ground 7:** Claim 49 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032), in further view of Nikon (EX1043);
- **Ground 8:** Claims 50 and 51 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032), in further view of Nikon (EX1043) and Skafte-Petersen (EX1048);
- **Ground 9:** Claim 52 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032), in further view of Sands (EX1046);
- **Ground 10:** Claim 53 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032) in further view of Sands (EX1046) and Brooker (EX1040);
- **Ground 11:** Claims 55, 57, and 58 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032), in further view of Wessels (EX1050);

- **Ground 12:** Claim 61 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032) in further view of the skill and knowledge of a POSA; and
- **Ground 13:** Claim 62 over the combination of Zhuang (EX1003), Cai (EX1004), and Shen (EX1032) in further view of Niman (EX1044) and/or Winer (EX1051).

IV. UNDERSTANDING OF THE GOVERNING LAW

16. I am a scientist, principal investigator and instructor by trade, and the opinions I express in this declaration involve the application of my scientific knowledge and experience to the evaluation of the claims of the '554 Patent and the impact of certain prior art on the '554 Patent. I am not a lawyer and have not been trained in the law of patents. Therefore, I have requested the attorneys from Weil, Gotshal & Manges, who represent NanoString, to provide me with guidance as to the applicable patent law in this matter. The paragraphs below express my understanding of how I must apply current legal principles related to patent claim construction and validity to my analysis.

17. It is my understanding that when interpreting the claims of the '554 Patent, I must do so from the perspective of one of ordinary skill in the art at the relevant priority date. I have been asked to assume that the '554 Patent has a priority date of November 3, 2015. I have not, however, made an independent

determination whether the '554 Patent is entitled to that priority date. My analysis does not change if the '554 Patent is entitled to a later priority date.

18. It is my understanding that in determining whether a patent claim under *inter partes* review before the Patent Trial and Appeal Board (PTAB) is unpatentable in view of prior art, the PTAB uses the *Phillips*¹ standard for claim construction used in civil courts, which is to say that the claims must be given the meaning that the terms would have had to a person of ordinary skill in the art at the time of the priority date of the '554 Patent consistent with the specification and file history of the '554 Patent.

19. For the purposes of this review, it is my opinion that each claim term can be easily understood by the skilled artisan without further construction.

20. I understand that a claim is unpatentable under 35 U.S.C. § 103 if the claimed subject matter as a whole would have been obvious to a POSA at the time of the alleged invention, which I have been instructed to treat at present as November 3, 2015. I also understand that an obviousness analysis takes into account the scope and content of the prior art, the differences between the claimed subject matter and the prior art, and the level of ordinary skill in the art at the time of the invention.

¹ *Phillips v. AWH Corp.*, 415 F.3d 1303, 1313 (Fed. Cir. 2005).

21. I understand that a patent claim composed of several elements is not proved obvious merely by demonstrating that each of its elements was known in the prior art. It has been explained to me that three requirements must be satisfied for a combination of prior art references to render obvious a claimed invention. First, the cited art must teach or suggest all the requirements of the invention as recited in the claims. However, I understand that a claim is obvious if a missing feature is a natural result of the combination of prior art elements. Second, the cited art, coupled with the knowledge generally available in the art at the time of the invention, must contain some suggestion or incentive that would have motivated the ordinary skilled artisan to modify a reference or to combine references. Third, the proposed modification of the cited art must have had a reasonable expectation of success, determined from the vantage point of the ordinary skilled artisan at the time the invention was made. I understand that it is impermissible to use hindsight to arrive at the claimed invention. My understanding is that to preclude hindsight in the obviousness analysis, which I understand to be improper, there must be evidence at the time of the invention of some reason to make the variation or combination. Furthermore, I understand that, to render a claim obvious, the prior art cannot be vague, but rather, must collectively guide a person of ordinary skill in the art towards the particular solution claimed. I understand that obviousness does not exist where the prior art discourages or teaches away from the claimed invention.

22. I understand that, in an obviousness analysis, only analogous art may be considered. Art is considered analogous if it is from the same field of endeavor as the patent. If it is not from the same field of endeavor, it may be considered analogous if it is reasonably pertinent to the particular problem with which the patent is involved. A reference is reasonably pertinent if, though in a different field, it would have commended itself to an inventor's attention in considering the problem the inventor is addressing.

23. I understand that in evaluating whether patent claims are obvious, objective indicia of non-obviousness can be considered, such as but not limited to, commercial success, long-felt but unsolved needs, praise, unexpected results, industry acceptance, failure of others, and skepticism by experts.

V. OVERVIEW OF THE '554 PATENT

24. The '554 Patent, including the challenged claims, is directed to methods of imaging nucleic acids, such as DNA or RNA, within a cell, along with an apparatus for carrying out this task. EX1001 at Abstract. To help provide context for the technological subject matter I discuss herein, I reproduce below claim 36 of the '554 Patent:

36. A system comprising:

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

25. Claim 36 of the '554 Patent is lengthy, but it can be viewed as having two basic parts. The first part consists of recited elements (a)-(e), and the second

part consists of software instructions associated with element (e), which refers to a “computing system.”

26. As to the first part of the claim that is set forth in elements (a)-(e), these elements recite what someone of skill in the art would understand are nothing more than standard, well-known components for optical imaging of biological samples, such as a microscope stage, sample holder, temperature control system, light source, objective lens, and computing system. Along these lines, the patent explains that various imaging techniques contemplated by the '554 Patent include three dimensional structured illumination, selective planar illumination microscopy (SPIM), light sheet microscopy, emission manipulation, volumetric imaging using pinhole confocal microscopy, volumetric imaging using aperture correlation confocal microscopy, volumetric imaging using volumetric reconstruction from slices, volumetric imaging using deconvolution microscopy, volumetric imaging using aberration-corrected multifocus microscopy, and volumetric imaging using digital holographic microscopy. *Id.* at 30:15-26.

27. The software instructions in step (e) pertain to imaging the sample multiple times to generate a collection of signals from the analytes in the sample, and then decoding the images to determine the identity and position of the analytes. As I explain herein, these were also known prior art operations. The '554 Patent, for instance, describes the use of fluorescence imaging techniques in which

fluorophores are used to fluorescently label oligonucleotides that are sequentially hybridized to target nucleic acids. *Id.* at 12:27-50, 15:19-67 (“the nucleic acids within the matrix can be interrogated using methods known to those of skill in the art including fluorescently labeled oligonucleotide/DNA/RNA hybridization”); *id.* at 16:25-27 (“The automated...three-dimensional imaging device may be used for detecting optical signals by fluorescence microscopy.”). Each fluorophore may have one of a number of colors, and these colors are detected in series or in parallel, with serial detection including rounds of detection once new probes are hybridized. *Id.* at 9:42-45 (“[T]he automated...three-dimensional imaging device described herein comprises a color multiplexer for detection of one or more distinct colors of light.”); *id.* at 9:61-63 (The “automated...three-dimensional imaging device...is used to detect two or more colors of light in serial or parallel.”).

28. All of these techniques were well understood in the art. As one example, and as I explain herein, the steps laid out in the software instructions in the claim essentially those used in the prior art MERFISH technique, which I discuss further below.

VI. PROSECUTION HISTORY OF THE '554 PATENT

29. I have reviewed the prosecution history for the '554 Patent, and include a brief summary below. I do not purport to be an expert in the process of patent

examination, but for the purposes of my opinions I wished to have a general understanding of the history of how the '554 Patent came to be issued.

30. The '554 Patent issued from prosecution of U.S. Application No. 17/395,534 that was filed on August 6, 2021. EX1001. It was subject to a rejection by the Patent Office on March 4, 2022. EX1035. Specifically, I understand that then-pending claims 9-38 were rejected as being obviousness in view of the prior art Keller (US 2015/0098126) (EX1042) and Callahan (US 2011/0216953) (EX1041) references. EX1035.009-.021.

31. Then-pending claim 38 read as follows, the “device of claim 9, further comprising, one or more microprocessors with software for automating or controlling image detection and acquisition of said optical system.” EX1037.004.

32. In rejecting claim 38, the Examiner relied on Keller for this limitation:

With regards to claim 38, Keller teaches a device further comprising, one or more microprocessors with software for automating or controlling image detection and acquisition of said optical system (data acquisition for the microscope system occurs at speeds that match the time-scales of the fastest processes of interest and with minimal time shifts between complementary views, wherein the system comprise an electronics controller and computational system to provide synchronized control of all mechanical components within the microscope with millisecond precision over long periods of time, and provides a robust pipeline

for simultaneous high-speed image acquisition with a plurality of detectors, paragraphs 44 and 123).

EX1035.014-.015.

33. In response, Applicant canceled claim 18, amended claims 9-17 and 19-38, and added new claims 39-45. EX1038.002-.003, .006-.013.

34. Applicant's amendment of claim 9 amended the preamble to recite a "system" rather than a "device" and added:

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:

determining a plurality of optically-encoded signals from a plurality of light signals, wherein said plurality of light signals is obtained from a plurality of read-out cycles, wherein said plurality of optically-encoded signals is associated with one or more nucleic acid molecules within said volume; and

determining three-dimensional positional information of said one or more nucleic acid molecules using said plurality of optically-encoded signals.

EX1038.006-.007.

35. Newly-added claim 45, which issued as claim 36, similarly recited a computer system for performing a method comprising:

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.

EX1038.012-.013.

36. Applicant argued for allowance of then-pending claim 9 (and claim 45 in effect) on the basis that:

Nothing in Keller teaches or discloses “at least one processor to perform a method comprising: determining a plurality of optically-encoded signals from a plurality of light signals, wherein said plurality of light signals is obtained from a plurality of read-out cycles, wherein said plurality of optically-encoded signals is associated with one or more nucleic acid molecules within said volume; and determining three-dimensional positional information of said one or more nucleic acid molecules using said plurality of optically-encoded signals,” as is recited in claim 9.

Callahan does not cure the above-mentioned deficiencies of Keller.

EX1038.002.

37. The Office then withdrew the obviousness rejection of the claims, noting in the Reasons for Allowance that:

[Keller] is silent with regards to a computing system comprising at least one computer readable storage medium having instructions that are executable by at least one processor to perform a method of determining a plurality of optically-encoded signals from a plurality of light signals obtained from a plurality of read-out cycles, the plurality of optically-encoded signals being associated with one or more nucleic acid molecules within a volume of a sample, and determining three-dimensional positional information of the one or more nucleic acid molecules using the plurality of optically-encoded signals. The secondary references of Callahan and Frisen are also silent with regard to the computing system as currently claimed.

EX1036.006.

38. The Examiner further noted that other references identified also failed to “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.”

EX1036.006-.007.

39. Thus, the upshot of the foregoing prosecution history is that claim 36 and those claims that depend from it (and claim 45 and its dependent claims in effect)

were allowed on the basis of the software instructions that are connected to step (e) of the claim.

40. With respect to the prior art that I rely upon in this declaration, the Cai reference (EX1013) is listed on the face of the '554 Patent (as Patent Publication 2015/0267251). EX1001 at code (56). I thus understand that Cai was submitted to the Patent Office during prosecution based on its inclusion in an Information Disclosure Statement (IDS) submitted by the Applicant on August 6, 2021. EX1034.014. But I am not aware of the Examiner giving Cai any analysis during prosecution. Neither Zhuang nor Shen were cited in an IDS, and neither were otherwise before the Patent Office during examination of the application that issued as the '554 Patent.

41. As I set forth in detail below, the combination of Zhuang, Cai, and Shen clearly renders obvious claim 36. It is my further opinion, accordingly, that the Examiner erred in failing to identify the combination of Zhuang, Cai, and Shen and to recognize that it taught systems, as claimed in the '554 Patent, that include the very elements the Examiner identified as not being taught in the prior art.

42. It is also my further opinion that, likewise, if other references before the Examiner during examination were to be determined to include the same or substantially the same teachings as Zhuang, Cai, and Shen it again would simply

prove that the Examiner erred in determining that the recited elements at issue were not taught by those other references that were before the Examiner.

43. As to additional references relied on in further grounds, i.e., Xiao, Ehrlicher, Tirichine, Brileya, Fang-Yen, Richard, Nikon, Skafte-Pedersen, Sands, Brooker, Niman, Wessels, and Winer, all discussed below, none were cited in an IDS or otherwise before the Patent Office during examination of the application that issued as the '554 Patent. EX1001 at code (56).

VII. TECHNICAL BACKGROUND AND STATE OF THE ART

A. Cellular Imaging

44. Long before 2015, there were numerous techniques available for fixed and live cell imaging, including stimulated emission depletion, confocal laser scanning microscopy, fluorescence photoactivation localization microscopy, stochastic optical reconstruction microscopy, structured illumination microscopy, and total internal reflection fluorescence microscopy. EX1018.002. Some of these techniques are capable of three-dimensional imaging. *Id.* at .009. These imaging techniques may utilize fluorescent probe molecules, which contain fluorophores that are excited by a light source and then emit red-shifted light several nanoseconds later. *Id.* The emitted light can be used to detect the probe and, thus, the target molecule that they are associated with, for example, by binding directly or indirectly to the target molecule.

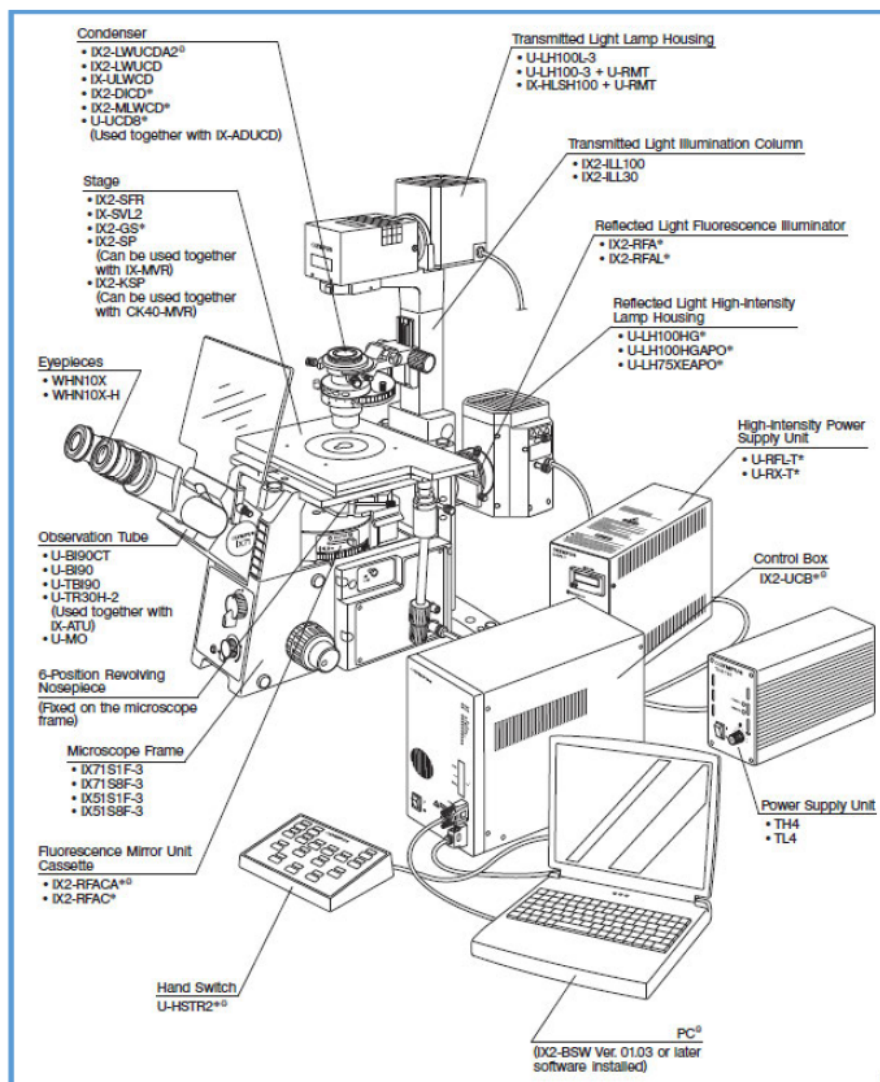
45. Well before 2015, microscopy was a well-established tool for three-dimensional cellular imaging. A POSA would have been aware of a variety of microscopy techniques, including structured illumination microscopy, STORM, PALM, laser scanning or spinning disk confocal microscopy, and light sheet based fluorescence microscopy (which can include selective plane illumination microscopy (SPIM) and digital scanned laser light sheet fluorescence microscopy (DSLM)). EX1019.003-.007. These techniques generally use some form of illumination to excite an optical label, and then an objective lens and detector to generate image(s) using emitted light from the excited labels attached to the probes. *Id.* By taking multiple images of optical-sections that are illuminated at different positions along the Z-plane, a three-dimensional image is generated. *Id.*

46. One commonly used microscope as of 2015 was the Olympus IX-71, depicted below. I am very familiar with this microscope and its equivalents through my experience in the field of microscopy and evaluation of various Olympus imaging platforms, and am aware that it had been available well before 2015. This microscope was released in the early 2000s and included a number of components and features that were either directly available from Olympus, or third parties, and would have been recognized as standard capabilities by a POSA. Extensive documentation that dates back prior to 2015 regarding this microscope is available. For instance, a product brochure for this microscope depicted it as follows:



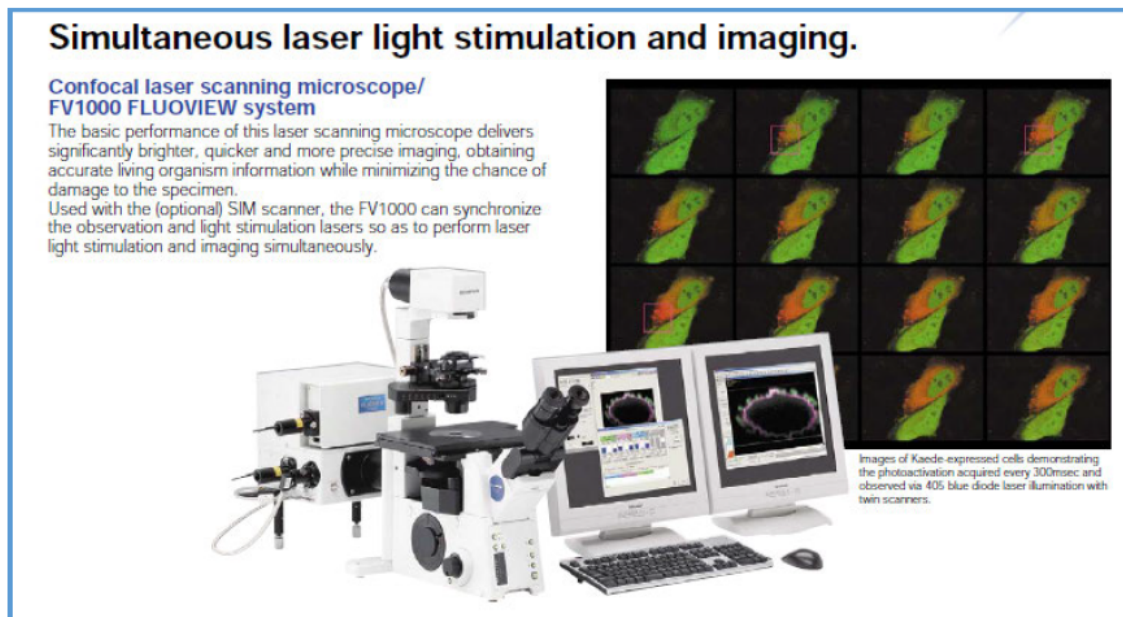
EX1033.003. This brochure is dated June 2009 and, as downloaded, includes metadata disclosing that it was modified in October 2009, which is consistent with the date of creation as having been prior to October 2009. EX1059.

47. As another example, below is a schematic of the Olympus IX71 from its product manual:



EX1005.008. The metadata for this manual, as downloaded, discloses a date of creation of April 2005, and that it was modified in February 2014, both of which are consistent with this microscope having been available prior to 2015. EX1058.

48. As another example, shown below is an image from another product brochure for the related IX81 microscope showing that the 3D confocal laser scanning system also can be used for imaging using laser light stimulation for cell fluorescence imaging:



EX1053.023. This brochure was also prepared no later than 2005, as confirmed by the metadata, as downloaded, showing it was modified in 2005. EX1060.

49. As reflected in the images above, the IX71/IX81 microscope models included several standard features that were common to all microscopes. For example, the microscope included a flat stage that could hold a sample and was capable of X-Y lateral motion and Z-axis up/down motion, a light source to illuminate the sample, and optical equipment, such as an objective lens, for visualizing the sample. *See generally* EX1005.

50. A POSA would have understood that this system, like many others of the time, could be used for 3D imaging. For instance, as one brochure explains, the Olympus IX-71 could be used to generate 3D images using, for instance, spinning disk confocal microscopy. EX1033.015. Software packages were

available for use with the system that were specifically for the purpose of visualizing specimens in 3D. *See id.* at .012 (“For enhanced spectral resolution of multichannel images, a spectral unmixing tool is incorporated, as are tools for fluorescence image evaluation, such as co-localisation. cell^F also features Z-sectioning and image acquisition at different focal positions to visualise specimens in 3D.”).

51. As also depicted in the above images, microscopes such as the Olympus IX-71 could be connected to computing systems as of 2015, and earlier, e.g., laptop computer, as depicted. EX1005.008. It was common for microscopic imaging systems to be automated, and many such automated systems were commercially available. *See* EX1022.001. Automation of microscopic imaging systems can include automation of focus, X-Y stage positioning, image capture and microscope apparatus, and/or automation of image analysis. *See generally id.*; EX1032.002-.003. Fig. 1 (discussed below in Section VII.C.). Such automation can enhance experimentation and provide an efficient system that reduces labor and consumes fewer laboratory resources. EX1022.001. Of particular interest, this enabled the automation of data acquisition and analysis, which is described further in Section VII.C.

52. While microscopes came standard with a variety of features, it was common to make additional modifications to microscopes to permit the study of

specific sample types. Some of these features may include different types of illumination sources, such as LED or laser sources; a temperature control apparatus to regulate sample temperature; fluidics systems capable of delivering reagents to and aspirating reagents from a sample; and custom or purpose compatible lenses and filters to control wavelengths of light illuminating a sample and being imaged. *See generally* EX1003.011 (describing a homebuilt microscope around an Olympus IX-71 body); EX1004 at 27:22-24 (describing systems having temperature control, as evidenced by steps conducted at different temperatures).

53. As set forth in the Olympus IX-71 manual and brochures, the Olympus IX-71 itself could be used with a wide variety of optional components and options. *See generally* EX1005; EX1053; EX1033.

54. One such component type that could be used with the Olympus IX-71 would allow for “precise and stable” control of “Specimen temperature.” “The unique multi-port system and modular motorisation allow maximum flexibility – enabling you to readily perform experiments today and tomorrow.” EX1033.004, .009-.010, .015. “For the greatest flexibility in culture vessel fitting, heating plates are available which provide direct heating and temperature control from below the sample.” *Id.* at .015. “For longer experiments, stage incubators can keep the environment inside a laboratory dish completely stable. Olympus offers several

types of stage incubators.” *Id.*; *see also* EX1053.021 (“This thermoplate maintains the cell specimen container temperature at 37°C.”).

55. Likewise, there were components that allowed for the “[u]se [of] two light sources simultaneously.” EX1053.010. The “IX71/IX81 frames are equipped with input/output ports for a wide variety of light sources and detectors.” EX1033.009. For instance, the Olympus IX-71 microscope product literature explains that “[t]otal internal reflection fluorescence microscopy enables the investigation of surfaces with extremely high Z-resolution and without interfering background. Illumination combiners for up to three lasers and the MT20 illumination system allow fast switching (1 ms) between the different light sources and thus combined TIRF and widefield applications.” *Id.* at .013.

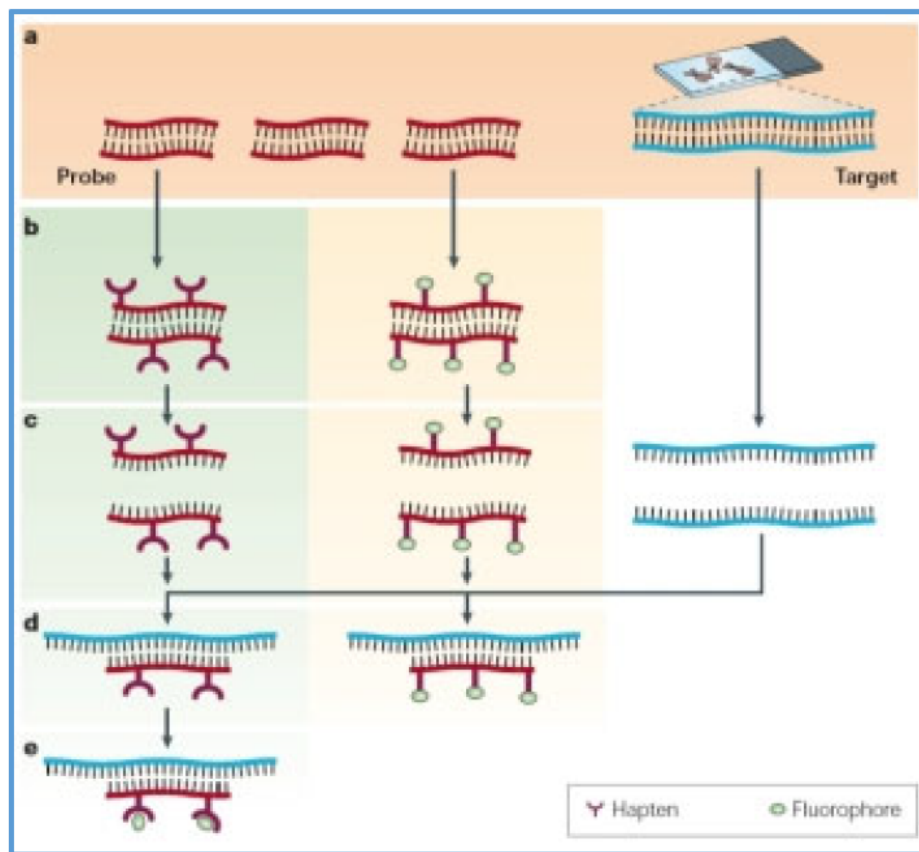
B. Microscopy Techniques For Imaging Nucleic Acids In Cells

56. As I explain above, microscopy had been a widely-used tool for the study of biological molecules decades before 2015. The location of nucleic acids, including messenger RNAs that are expressed and encode proteins, in cells and tissues provide useful information for biological research and medical diagnostics. Below, I summarize some of the approaches that were used with microscopy to analyze nucleic acids in cells.

57. Fluorescence *in situ* hybridization (FISH), sequential fluorescence *in situ* hybridization (seqFISH), and multiplexed error robust fluorescence *in situ*

hybridization (MERFISH) are three techniques that can be used to identify the nucleic acids that are expressed, the amounts in which they are expressed, and their location within cells and/or tissues.

58. Fluorescence *in situ* hybridization (FISH) relies on using fluorescent probes, *e.g.*, nucleic acids coupled to fluorescent labels, to generate images of cells or tissues. EX1020.002-.003 ; EX1017. The following image illustrates the basic concept underlying FISH:



EX1017 at Fig. 1. The probes that are used in FISH are shown in red and cellular target in blue in panel **a** above. The probe nucleic acids (red) that are used to detect

targets (blue) are shown in panels **b** and **c** above, and consist of nucleic acid molecules coupled to labels that can be detected. *Id.* at Fig. 1 (legend). The sequence of the nucleic acid probe—either RNA or DNA—is complementary to a target sequence and can hybridize—via base pairing—with RNA or DNA having the target sequence in the cell(s) or tissue to be imaged. *Id.* at .001. Then, the target nucleic acids can be detected by virtue of the attached label, which is often a fluorophore. FISH is used, “among other things, to detect and localize the presence or absence of specific DNA sequences or RNA targets.” EX1004 at 14:29-31. FISH is “widely known and practiced by persons having ordinary skill in the art.” *Id.* at 14:27-29.

59. seqFISH (sequential fluorescence *in situ* hybridization) is a variant of FISH that uses fluorescently-labeled nucleic acid probes with sequential rounds of hybridization and fluorescent imaging to capture three-dimensional images of cells or tissues. EX1020.002-.003. In a nutshell, seqFISH uses multiple labels (and colors) and hybridization rounds to identify each target whereas traditional FISH uses only a single label and hybridization round per target. Thus, applying sequential hybridization of the probes to target nucleic acids of interest and imaging, with detection of fluorescence signal at different locations, provides a *series* of signals for each location where hybridization occurs, and the series of signals (“barcodes”) can then be used to identify the target mRNA or DNA, which then also

provides two- or three-dimensional spatial information about the target mRNA or DNA in the cell or tissue. *Id.* at .003-.004, .018. Computer software is used to decipher and match the barcode fluorescence signal sequences associated with their respective mRNA or DNA sequences. *Id.* at .020.

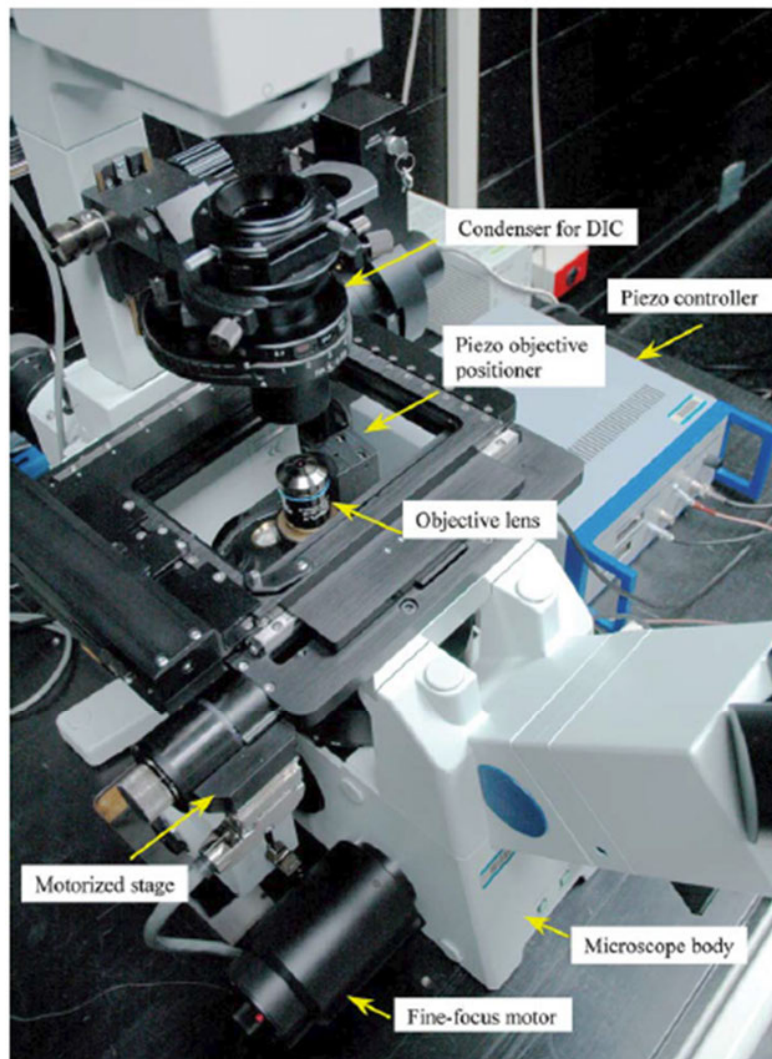
60. Another variant of FISH, multiplexed error robust fluorescence *in situ* hybridization (MERFISH), like FISH and seqFISH, images cell and tissue by targeting individual RNA molecules by hybridization with encoding probes which include multiple read-out sequences that can sequentially be hybridized to fluorescent probes. EX1021. MERFISH allows simultaneous hybridization with many different probes and followed by sequential imaging and identification of hundreds to thousands of different RNA species to be mapped from the same specimen, providing spatial context. *Id.* at Abstract. Ultimately, MERFISH utilizes a combinatorial barcoding and imaging approach with sequential hybridization followed by computational image analysis. *Id.* at .003-.005, .008. MERFISH operates according to the same general principles of hybridization as FISH and seqFISH, but includes additional features (which are not important for this Petition) to correct for errors and extend the number of unique target sequences that can be measured.

C. Automation of Imaging Systems

61. Long before 2015, the use of automated microscopic imaging systems was common, and many systems were commercially available. EX1022.001 (disclosing such in 2011). Automation of imaging systems can include automation of the image capture and microscope apparatus, or automation of image analysis. *See generally id.* Such automation can enhance experimentation and provide an efficient system that reduces labor and consumes fewer laboratory resources. *Id.* Also, automated image processing techniques have “greatly facilitated the task of analyzing cell activity” (*id.* at .002) and can “greatly decrease[] background noise and [] enhance resolution” (EX1018.006).

62. One example was the use of automation to maintain focus during time-lapse studies, which is valuable to ensure that clear images can be taken at any point during the lapse. Also long before 2015, as early as 2006, the problem of focus fluctuations was known. Potential causes of such fluctuation are that: (1) the substrate supporting the specimen is not flat; (2) thermal, or temperature, fluctuation causes the distance between the sample and the optical elements to drift over time; and (3) fluid flow and exchange of medium in cell perfusion experiments cause vibrations. EX1032.001. By 2006, however, commercially available systems were able to address these issues. Shen, for example, teaches the use of an Olympus IX-71 microscope, discussed earlier, with an “autofocus module.” *Id.*

at .003. As depicted below, the microscope has several automation features that allow autofocusing.



Id. at Fig. 1 (depicting, *inter alia*, a microscope with a motorized stage, piezo objective position, piezo controller, and fine-focus motor).

63. Shen describes five components “needed to carry out digital autofocus in a microscopy scan”:

- A microscope capable of differential interference contrast (DIC) imaging
- Hardware to precisely control the spacing between the objective and the specimen
- A motorized x-y stage with linear encoding feedback accuracy
- An equivalent of a computer with a Pentium 4 2.4-GHz central processing unit (CPU), 2-GB random access memory (RAM), and minimum of two peripheral component interconnect (PCI) expansion slots
- A scientific grade digital camera that can interface with a computer

Id. at .002.

64. Automation of the microscope and imaging system, such as what is described by Shen, can enhance experimentation and provide an efficient system that reduces labor requirements, consumes fewer laboratory resources, and increases data content per experiment, which greatly enhances cell science. EX1022.001.

65. Automated image processing techniques have “greatly facilitated the task of analyzing cell activity” (*id.* at .002) and can “greatly decrease[] background noise and [] enhance resolution” (EX1018.006). A number of commercial and open-source packages exist for automated image processing, including ImageProPlus, MetaMorph, Northern Eclipse, Cell-Profiler, and ImageJ. EX1022.005.

66. More generally, image processing software was well known long before the priority date for the '554 Patent. For example, ImageJ is a popular open source image processing software capable of running on numerous platforms. EX1023.001-.002. ImageJ can read most common image formats, can directly acquire images from cameras, and supports image processing operations such as convolution, edge detection, Fourier transform, histogram and particle analysis, editing and color manipulation, dilation, erosion and closing of structures, visualizations, three-dimensional plotting, and volume rendering. *Id.* at .002. The use of NIH Image, the predecessor to ImageJ, for such work was known as early as the 1980s, with the version of ImageJ in use in 2012 being developed in 1997. *Id.* at .001; Schneider (EX1047) at .002-.003, Table 1.

67. Yet another example of popular open source software capable of image processing is Matlab, a programming platform designed for analysis and visualization of data. By 2015, there were “toolkits” available for use in cell imaging. *See generally* EX1024; EX1025. These “toolkits” could automatically receive images from microscopy systems and perform analysis on those images to construct three-dimensional representations. *See, e.g.*, EX1024.014.

VIII. PERSON OF ORDINARY SKILL IN THE ART

68. In my opinion, a person having ordinary skill in the art relevant to the '554 Patent as of November 3, 2015 (the earliest possible priority date of the '554

Patent) would have had a Ph.D. in Molecular, Cellular, or Developmental Biology and/or Biophysics, Bioengineering, Biochemistry, or a related discipline with 2 years of experience in the field of fluorescence imaging of biological materials, including cells and tissues, and analysis of image data.

69. A POSA would have understood the fluorescence imaging technologies themselves, including fluorescent label technology, the use of microscopes to collect and record images, and the processing of images and image data to obtain 2D or 3D spatial information relating to the imaged cells or tissues. A POSA would have had experience and/or knowledge of optical systems (such as microscopes), three-dimensional and volumetric imaging technologies, including microscopy, microfluidics, and related computational analysis and image-based software programs for acquiring and analyzing 3D images, including generating 3D spatial information, and processing and quantification relating to signals received from labeled materials. A POSA also would have had experience and/or knowledge of detectable labels, including fluorescent labels, used for labeling nucleic acids and the use of such labeled nucleic acids to identify and characterize other nucleic acids within a sample based on the *in situ* hybridization of the labeled nucleic acid to nucleic acids of interest having complementary sequence, e.g., sequence to which the labeled nucleic acids will bind through hybridization based on complementary sequences.

70. A POSA in the field of fluorescence imaging would also have had some familiarity with state-of-the-art gene amplification and sequencing technologies. In particular, a POSA would have had some familiarity with these technologies due to the overlap in the underlying fundamentals involving hybridization of nucleic acids and fluorescent labels (and the detection thereof), obtaining image data of fluorescent labeled material, and analysis of data obtained from detection and/or imaging of fluorescent labeled material. Further, to the extent such related technologies were not considered to be within the same field, a POSA would have been aware of these technologies, and recognized that the methods and techniques used in these related technologies would have been useful to address the problems that arise in using fluorescently-labeled nucleic acid probes with *in situ* hybridization, imaging using such, and the processing of resulting images and image data to obtain spatial information relating to the imaged cells or tissues.

IX. THE CITED PRIOR ART

71. While this IPR relies on Zhuang, Cai, and Shen for independent claim 36 and many dependent claims (with additional references for other dependent claims), the background art discussed throughout this declaration reflects the general knowledge of a POSA before November 3, 2015, which I am informed must be taken into account when evaluating obviousness.

A. Zhuang (EX1003)

72. Zhuang is a scientific paper (only published online), with a research article summary (published in print form, dated April 24, 2015), where both the research article summary and article were published online on April 9, 2015; Chen et al., “Spatially resolved, highly multiplexed RNA profiling in single cells,” *Science* 348(6233): 412 (2015) (Research Article Summary) (with the full article available at <http://dx.doi.org/10.1126/science.aaa6090>, including as a downloadable pdf version at <https://www.science.org/doi/epdf/10.1126/science.aaa6090>). EX1003.001, .015. I understand that Zhuang was publicly available as of April 9, 2015, because that is the date the article states it was published online and, further, it was at least available as of the date of the printed publication of the Research Article Summary, dated April 24, 2015, as, in my experience, linked articles are publicly available as of the date that links are published in print or other on-line articles. EX1003 also appears to be an authentic copy of Zhuang in pdf form downloaded from the link(s) cited above.

73. Zhuang describes MERFISH, which, as I note above, stands for Multiplexed Error-Robust Fluorescence *In Situ* Hybridization. It is a highly multiplexed imaging method that ultimately allows imaging and spatial determination of multiple unique nucleic acid populations by individually and

combinatorially labelling (barcoding) them with fluorescent probes. EX1003.002 (Abstract), .003.

74. Zhuang's method involves performing multiple rounds of hybridization and imaging of the target nucleic acids in a sample. EX1003.003. Zhuang relies on encoding probes that are hybridized to target nucleic acids where the encoding probes also include sequence that, in each round, are hybridized with complementary fluorescent probes. *Id.* The sample is then imaged with the help of a microscope and fluorescent spots corresponding to individual nucleic acids are clearly detected. *Id.* at .003, .011. The sample region is repeatedly imaged with different sets of probes in each round. *Id.* at .003, .011-.012. With each round, after imaging, the probes are bleached to prevent them from interfering with subsequent imaging cycles. *Id.* Over multiple cycles, each fluorescent spot in the final image is decoded to identify the corresponding target nucleic acids and their position in the sample. *Id.* Based on the presence or absence of the fluorescence signal in each round, one can identify and spatially localize the presence or absence of a target nucleic acid molecule.

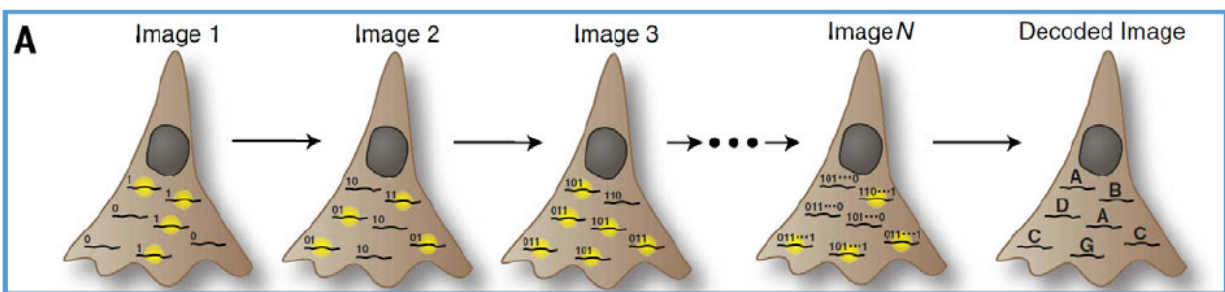
75. Zhuang teaches the use of an automated system constructed around a modified version of the Olympus IX-71 microscope (which I discuss in detail above) comprising various components to carry out the automated steps in MERFISH imaging. EX1003.003. Zhuang discloses the use of a flow chamber that holds

the sample, which can include cell(s) or tissue. *Id.* at .011, .012. The flow of fluid and reagents to carry out the MERFISH process through this chamber is controlled by a fluidics system. *Id.* at .002, .011. This fluidics system includes “three computer-controlled eight-way valves (Hamilton, MVP and HVXM 8-5) and a computer-controlled peristaltic pump (Rainin, Dynamax RP-1)” to achieve uninterrupted fluid transfer to the sample for sequential cycles of hybridization, imaging and bleaching. *Id.* at .011-.012. Fluid reagents include nucleic acid probes for hybridization that are fluorescently labelled. *Id.* These fluorescently labelled probes provide fluorescence light signals that are captured in the imaging process. *Id.*

76. Zhuang further discloses the use of an optical system, i.e., a microscope, configured for sequential imaging of samples. Specifically, as I note above, Zhuang discloses that a sample is “imaged on a home-built microscope constructed around an Olympus IX-71 body and a 1.45 NA, 100× oil immersion objective and configured for oblique incidence excitation.” EX1003.011. Zhuang’s optical system also is comprised of light sources for excitation of the nucleic acid probes. *Id.* (“Illumination was provided at 641...nm...using solid state lasers (MPB communications, VFL-P500-642...) for excitation of [the] Cy5-labeled readout probes...”). Further, Zhuang’s system utilizes cameras and emission filters to detect the various wavelengths of light emitted, including that

emitted by the fluorophore-labeled nucleic acid probes. *Id.* (Fluorescence was separated with a QuadView (Photometrics) by using the dichroics T560lpxr, T650lpxr, and 750dcxxr (Chroma) and the emission filters ET525/50m, WT59550m-2f [sic, WT595/50m-2f], ET700/75m, and HQ770lp (Chroma) and imaged with an EMCCD camera (Andor, iXon-897)).

77. Zhuang teaches the determination of multiple nucleic acid species in a sample with the plurality of images taken at each round. For example, in Figure 1(A), Zhuang illustrates use of a two-step labeling scheme with hybridization of RNA molecules with a set of fluorescently labelled encoding probes that include RNA targeting sequence and flanking readout sequences followed by hybridization of fluorescently labelled probes to the readout sequences and imaging in each successive round. *Id.* at Fig. 1(A). Fluorescent signal at a location, indicating hybridization, is coded as 1 and lack of a signal, indicating no hybridization, is coded as 0. With each round, a new set of fluorescently labelled probes are either hybridized to encoding probes that are hybridized to nucleic acids of interest in the sample or not, as reflected in the figure by the growing string of 1 or 0 values over successive rounds. This repeated hybridization and imaging ultimately results in collection of sufficient information of the identity and location of the RNA species present to identify their identity and location, as depicted in the rightmost image with RNAs at different locations labeled as A, B, C, D, and G.



Id. at Fig. 1(A).

78. Furthermore, Zhuang teaches that its entire system is “fully automated, so that imaging and fluid handling were performed for the entire experiment without user intervention.” EX1003.011. As a result, Zhuang discloses the use of a computer that automates the fluidics and optical system. Zhuang’s computing system is configured to control imaging of the sample, for example by using super-resolution imaging techniques, and analyzing the average number of light signals, for example by correlating the fluorescent light signals to individual nucleic acid species, to reveal information on the identity and location of these nucleic acid molecules in the sample.

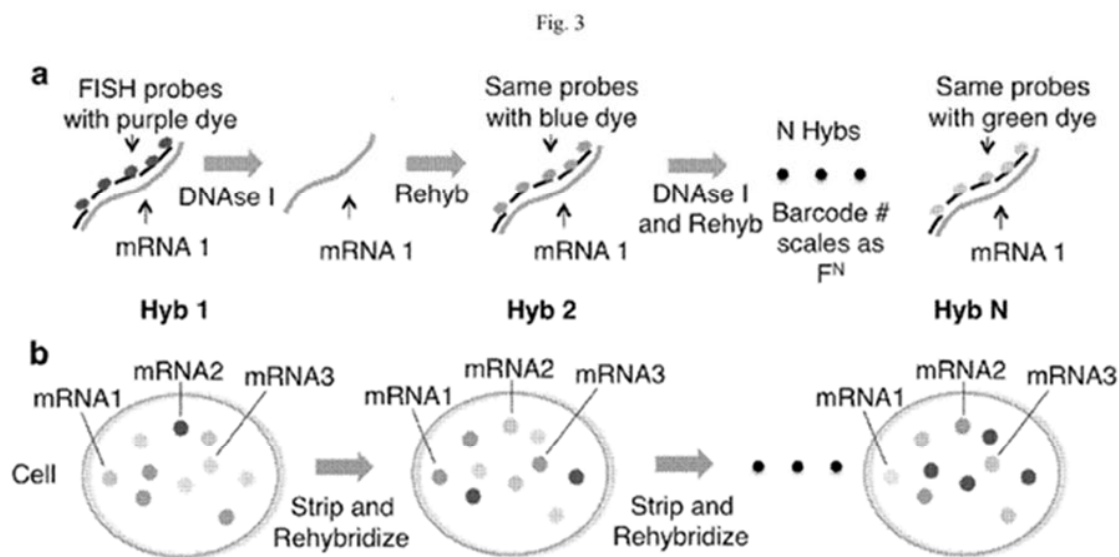
B. Cai (EX1004)

79. Cai is entitled “Multiplex Labeling of Molecules by Sequential Hybridization Barcoding” and issued as U.S. Patent No. 10,457,980, on October 29, 2019, from Application No. 14/435,735, the national phase of PCT/US2014/036258, was filed on April 30, 2014. EX1004 at codes (10), (12), (21), (22), (45), (54),

(86). The PCT application was published as WO2014/182528 on November 13, 2014. *Id.* at code (87).

80. Cai teaches systems and methods for seqFISH. As I describe above, seqFISH is a variant of FISH that uses fluorescent probes to label target nucleic acids in capturing three-dimensional images of cells or tissues using sequential rounds of fluorescent probe hybridization and fluorescence imaging. *See supra* Section VII.B.

81. Cai, broadly, discloses two schemes to accomplish such sequential rounds of hybridization and imaging. In the first scheme, depicted in Figure 3 of Cai, “each transcript is targeted by a set of multiple, for example, 24 FISH probes, labeled with a single type of fluorophore. The sample is imaged and the FISH probes are removed by enzymatic digestion. Then the mRNA is hybridized in a subsequent round with the same FISH probes, but now labeled with, in some cases, a different dye.” EX1004 at 41:13-30.

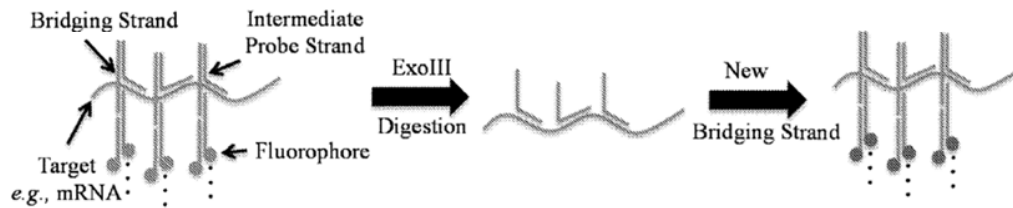


Id. at Fig. 3. This scheme can be understood as a direct targeting scheme. In other words, as depicted in the figure and described in the patent, a probe specific to an mRNA of interest with a fluorophore directly affixed to it binds to the mRNA, is stripped off entirely, and a new probe with a fluorophore is bound. Successive rounds generate a barcode unique to that RNA molecule. *Id.* at 41:13-30, Fig. 3.

82. In the second scheme, oligonucleotide probes labeled with specific fluorophores are hybridized to corresponding intermediate oligonucleotides that are themselves that are bound to target mRNA or DNA populations *in situ*, and remain bound to the target over successive rounds. *Id.* at 26:21-65. This is depicted, for example, in Figure 21(a).

Fig. 21

(a)



Id. at Fig. 21(a). In this figure, there is an intermediate probe strand that binds to a target mRNA of interest. Labeled probes are then hybridized to that intermediate probe strand. *Id.* at 26:21-65, Fig. 21(a).

83. Between rounds, the labeled probes are stripped from the intermediate probe strands before re-hybridization of different labeled probes to the same intermediate probe strands, while the intermediate probe strands remain bound to the target nucleic acids of interest. *Id.* at 26:21-65. In each round of hybridization and imaging, the color of fluorescence signal of each bound probe is detected to obtain information as to the sequence present and at what location within the cell or tissue. *Id.* The series of fluorescent signals reflecting the binding, a sequential barcode, is then used to uniquely identify the target mRNA or DNA, and, when data is collected over the z-axis, three-dimensional spatial information about the target mRNA or DNA within the sample cell or tissue. EX1004 at 37:28-50. Computer

software is used to match the fluorescence signal sequences associated with different mRNA or DNA sequences. *See, e.g.*, EX1004 at 46:18-33.

84. The probes in both schemes are able to diffuse into a sample and hybridize with nucleic acid molecules of interest. *Id.* at 47:57-61. The detectable labels can include a fluorophore or fluorescent dye, wherein the “detectable signal is a certain color.” *Id.* at 4:40-45, 15:33-45; *see also id.* at 16:26-37 (providing exemplary fluorescent dyes).

85. The sequence of light signals from these labels are used to determine barcodes for each position within the sample, corresponding to different nucleic acids of interest, based on the sequence of hybridization to corresponding labeled probes.

Each target, after rounds of contacting and imaging, has its own unique combination of labels (sequential barcoding), so that information, e.g., quantitative and/or spatial information, can be obtained for a target. For example, when fluorophores are used to label detectably labeled oligonucleotides, after N steps, a target would have a sequential barcode of $F_1 F_2 \dots F_N$, wherein F_N is the color of the fluorophore used for the target in the n -th imaging. One target can be differentiated from another by a difference in their barcodes (e.g., RedRedBlueRed compared to RedRedRedBlue).

Id. at 15:22-32.

86. Cai also discloses particular systems for use with seqFISH. In a section titled “Hardware,” Cai describes an exemplary system for seqFISH. EX1004 at 45:32-62. The “automated system” is described as a sequencer that “consists of or comprises an automated epi-fluorescence microscope to perform the imaging and an automated fluidics system to perform the sequential hybridizations.” *Id.*

87. In yet another embodiment, exemplified by Figure 17, seqFISH can be performed on a light sheet microscope. *Id.* at 42:20-21, 44:28-33 (disclosing light sheet microscopy for mapping mouse brains), 46:34-48 (“[light sheet microscopy] provides methods for measuring thick tissues or organs”), 21:48-58 (“an imaging step provides three-dimensional spatial information of a target”). The microscope of Figure 17 uses a two-dimensional plane of light sheet having an x-axis and y-axis that illuminates the sample perpendicular to the detection objective. *Id.* at 47:30-47.

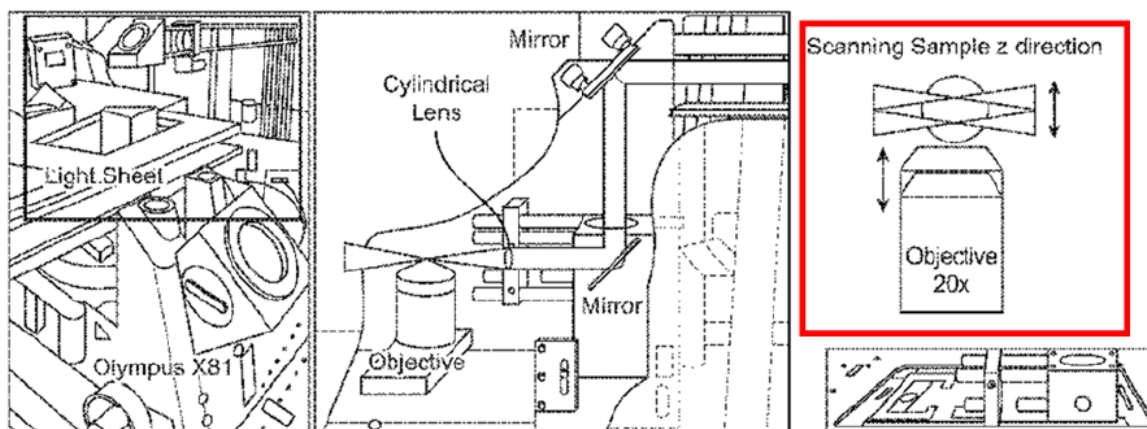


FIG. 17

EX1004 at Fig. 17 (emphasis added) (depicting scanning sample along Z-axis by moving a coupled laser and objective in Z-axis; both identified by double-headed arrows in figure).

88. The microscope is configured such that the sample can be scanned along the Z-axis relative to the two-dimensional light sheet such that the sample can be illuminated and measured in three dimensions. *Id.* at 47:43-45 (“the objective is moving synchronically with the illumination sheet, allowing scanning the sample along z-axis”). The microscope system includes a hybridization chamber where the three-dimensional sample is mounted. *Id.* at 47:45-47.

89. Cai also teaches automation of imaging technology, including “automated fluidics, image acquisition, and/or integrated analysis [that] can be independently and optionally combined with fast hybridization cycle time and imaging time,” and that “valves, stages, light sources, cameras and/or microscopes

are controlled through Micromanager,” a free software package supported by the NIH. *Id.* at 45:28-46:3.

90. Cai also discloses automated software for data processing. “Software packages for data analysis...in Python and Matlab” provide automated software capable of image analysis for three-dimensional cellular imaging. *Id.* at 46:16-33. In a section titled “Automation,” Cai teaches that automation of software “can be applied to efficiently scale up, for example, to map 100 genes and/or reduce human labor and/or errors,” and that “integrated analysis can be independently and optionally combined with fast hybridization cycle time and imaging time.” *Id.* at 45:22-31.

C. Shen (EX1032)

91. Shen is a chapter in the book series *Methods in Enzymology* (Elsevier Inc.) by Shen et al., published in 2006’ Shen et al., “Digital Autofocus Methods for Automated Microscopy,” *Methods in Enzymology* 414: 620-632 (2006)). EX1032.001. In my experience, such publications are available as of, or soon following, the copyright date. EX1032 also appears to be an authentic copy of Shen. The *Methods in Enzymology* series is a well-known and widely-disseminated resource, would have been known to a POSA, and evidences the knowledge of a POSA at or following its publication.

92. Shen teaches an “implementation of a robust autofocus system appropriate for using either air or oil immersion objectives in robotic imaging,” in other words, ways to image a sample using an autofocusing microscope system. EX1032.001. This is accomplished with hardware that “acquires a z stack of images for each focus attempt” and software that “determines the high-frequency content of each image in the stack and uses the results to determine a ‘focus index’ for each image.” *Id.* at .002.

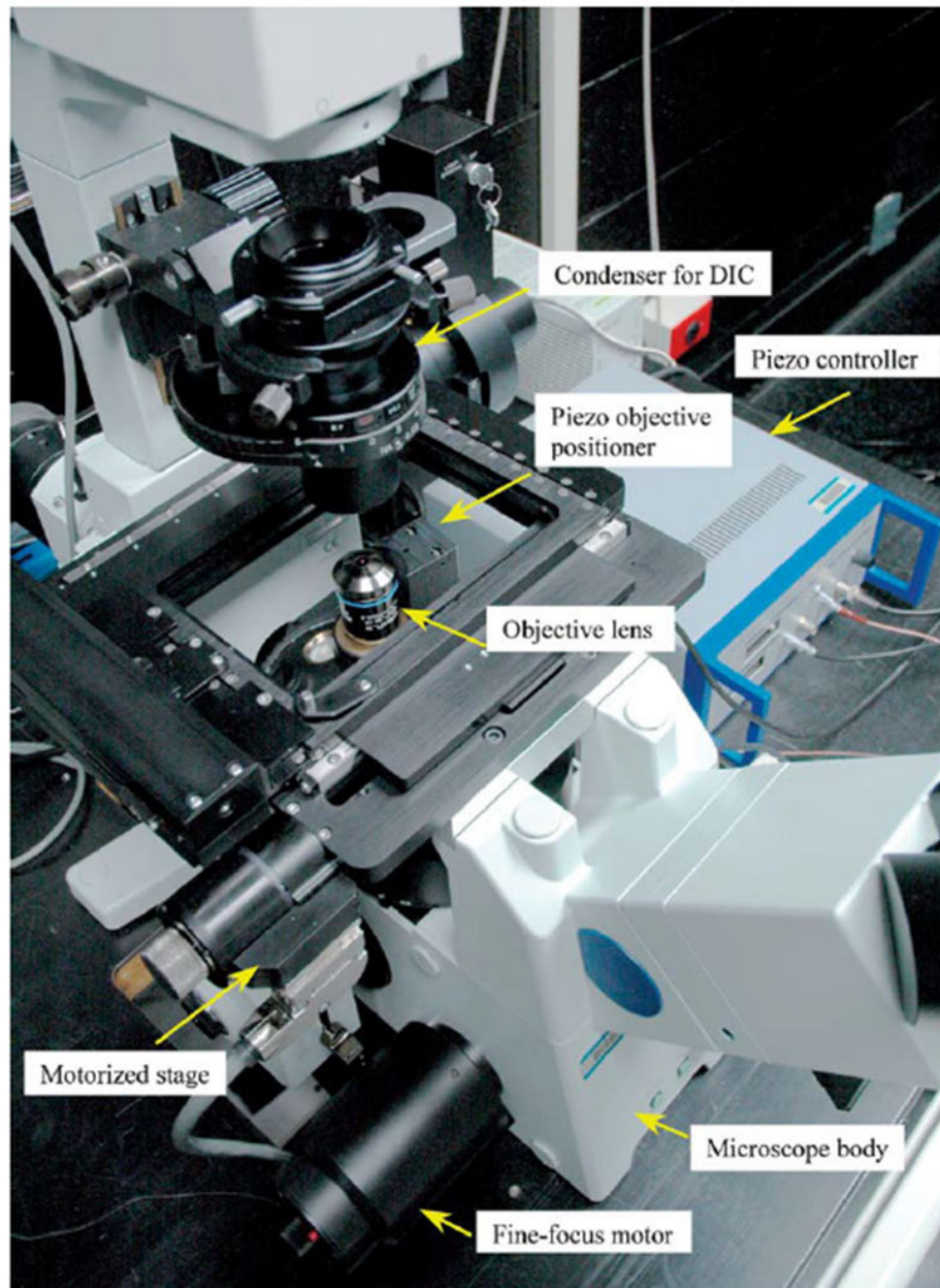
93. Shen lists five components needed to carry out digital autofocus in a microscopy:

- A microscope capable of differential interference contrast (DIC) imaging
- Hardware to precisely control the spacing between the objective and the specimen
- A motorized x-y stage with linear encoding feedback accuracy
- An equivalent of a computer with a Pentium 4 2.4-GHz central processing unit (CPU), 2-GB random access memory (RAM), and minimum of two peripheral component interconnect (PCI) expansion slots.
- A scientific grade digital camera that can interface with a computer

Id. As an example of an assembled system, Shen discloses the use of an Olympus IX71 inverted microscope with a fitted autofocus module, equipped to perform DIC imaging. *Id.* at .003. Shen describes DIC imaging as preferred because it

“attenuate[s] fluorescence, is easy to set up, and has greater high-frequency content.”

Id. Below is a figure depicting aspects of the described system:



Id. at Fig. 1. Figure 1 depicts the motorized stage, the fine focus motor to control z-axis positioning, an objective lens, a light condenser for DIC, a piezo objective

positioner (which is a device that provides linear motive force), a piezo controller that can be used to control the focus, and the microscope body.

94. While Shen relied on the use of a piezo motor—the PIFOC—for its autofocusing experiments, it teaches that other motors may be used. For example, one identified alternative is an “electric stepper motor attachment with linear encoded feedback” that is “installed on the fine focus knob of the microscope.” *Id.* at .004.

95. In addition to this hardware, Shen teaches software that “carries out the autofocus calculation instructions. It also controls the hardware, that is, stage movement, piezo movement, light path shuttering, and image acquisition via the camera.” *Id.* at .005.

D. Xiao (EX1027)

96. Xiao is a chapter in the Handbook of Single-Molecule Biophysics, published by Springer in 2009; Xiao, “Single-Molecule Imaging in Live Cells,” *Handbook of Single-Molecule Biophysics* 43-93 (2009) (Springer). EX1027.063. In my experience, such handbooks are available as of, or soon following, the copyright date. EX1027 also appears to be an authentic copy of Xiao. Xiao provides a comprehensive overview of single-molecule imaging in live cells, with particular focus on use of fluorescent labels and special considerations for live-cell imaging.

97. Xiao teaches the use of cell chambers for optimal cell growth during a prolonged imaging period, allowing growth or monitoring of live cells on the microscope stage. *Id.* at .098-.099. Xiao identifies Bioptechs FCS2 as a commercially available cell growth chamber and discusses its use, including that it can be used in a closed and open-dish format.

The FCS2 system can also be used for mammalian cells. Cells are first cultured on coverslips immersed in growth media as usual, and then the coverslip is assembled into the closed chamber for observation. *An open-dish format, in which the chamber is not sealed by the use of a top glass slide, is also popular in live-cell imaging.* The easiest open-dish format is essentially a culture dish with a coverslip mounted at the bottom. This format allows easy addition of chemical stimuli but, again, suffers from unregulated temperature and media evaporation during long experiments. One important consideration for mammalian culture on a microscope stage is that if the medium is not perfused with CO₂ during the experiment, a growth medium with its pH not dependent on CO₂ such as Leibovitz's L15 should be used.

Id. at .099 (emphasis added). Xiao identifies advantages of using the FCS2 system chamber in an open-dish format, that is, as an open flow cell, such as allowing easy addition of chemical stimuli. *Id.*

E. Ehrlicher (EX1028)

98. Ehrlicher is a chapter in the book series *Methods in Cell Biology* (Elsevier Inc.) published in 2007; Ehrlicher et al., “Chapter 21: *Optical Neuronal Guidance*,” *Methods in Cell Biology* 83: 495-520 (2007) (Elsevier Inc.). EX1028.001. In my experience, such publications are available as of, or soon following, the copyright date. EX1028 also appears to be an authentic copy of Ehrlicher. The *Methods in Cell Biology* series is a well-known and widely-disseminated resource in the field of cell biology, would have been known to a POSA, and evidences the knowledge of a POSA at or following its publication.

99. Ehrlicher describes methods for studying neurons that include laser irradiation and imaging of live neuronal cells using fluorescence microscopy. EX1028.002, .009, Fig. 2 (illustration of experimental setup), .011-.012 (discussing systems for maintaining live cells on microscope stage). Ehrlicher teaches that Biopetechs FCS2 Focht Live-Cell Chamber and objective heater could be used and that it offers an open or closed design. *Id.* at .012 (“We initially used a...commercially available system (Biopetechs FCS2 Focht Live-Cell Chamber and objective heater; Butler, Pennsylvania), which has excellent temperature control and offers an open or closed design.”).

F. Tirichine (EX1049)

100. Tirichine is a scientific paper published August 3, 2009; Tirichine et al., “3D fluorescent in situ hybridization using Arabidopsis leaf cryosections and isolated nuclei,” *Plant Methods* 5:11, 1-7 (2009). Tirichine states that it was published “3 August 2009.” EX1049.001. In my experience, such publications accurately identify the date of publication and are available online as of that date. EX1049 appears to be an authentic copy of Tirichine downloaded from <https://plantmethods.biomedcentral.com/counter/pdf/10.1186/1746-4811-5-11.pdf>.

101. Tirichine teaches a protocol for FISH performed on 3D isolated nuclei and thin cryosectioned leaves of *Arabidopsis thaliana*. In particular, Tirichine discloses the use of a water immersion objective as part of a confocal microscope system to capture fluorescent images as part of the FISH protocol. EX1049.003.

G. Brileya (EX1039)

102. Brileya is a scientific paper published online December 15, 2015; Brileya et al., “Biofilm growth mode promotes carrying capacity and community stability during product inhibition syntrophy,” *Frontiers In Microbiology* 5:693, 1-13 (2014). EX1039.001. Brileya states that it was published “15 December 2014.” *Id.* In my experience, such publications accurately identify the date of publication and are available online as of that date. EX1039 appears to be an

authentic copy of Brileya downloaded from
<https://www.frontiersin.org/articles/10.3389/fmicb.2014.00693/full>.

103. Brileya investigated biofilms using FISH to determine the relative biovolume of different cell types in a sample. EX1039.003. In particular, on intact, unscrapped biofilm samples, 3D-FISH was used to determine colocalization patterns of various biomarkers. *Id.* Imaging for 3D-FISH was performed using a water dipping objective. *Id.*

H. Fang-Yen (EX1029)

104. Fang-Yen is a scientific paper published online January 14, 2011; Fang-Yen et al., “Video-rate tomographic phase microscopy,” *Journal of Biomedical Optics* 16(1): 011005-1—011005-5. EX1029.001. Fang-Yen states that it was published January 14, 2011. *Id.* In my experience, such publications accurately identify the date of publication and are available online as of that date. EX1029 appears to be an authentic copy of Fang-Yen downloaded from <https://www.spiedigitallibrary.org/journalArticle/Download?urlId=10.1117%2F1.3522506>.

105. Fang-Yen describes tomographic phase microscopy (TPM) to measure the “3-D refractive index distribution of cells and tissues by combining the information from a series of angle-dependent interferometric phase images.” EX1029.001 (Abstract). Fang-Yen, as a part of its method, teaches cell imaging

by utilizing various standard elements in optical microscopy, including “a manual syringe or a syringe pump (Harvard Apparatus (Holliston, Massachusetts) PHD 22/2000).” *Id.* at .002. Fang-Yen discloses the use of this syringe pump to inject culture medium into a “flow chamber [custom made or Biopetechs (Butler, Pennsylvania) FCS2].” *Id.* It further discloses that “[u]sing the syringe pump, the hyperosmotic solution was injected into the chamber at a rate of 1.5 mL/min.” *Id.*

I. Richard (EX1030)

106. Richard is a scientific paper published in 2011 (online December 24, 2010); Richard et al., “Cellular mechanisms by which lipoic acid confers protection during the early stages of cerebral ischemia: A possible role for calcium,” *Neuroscience Research* 69: 299-307 (2011). EX1030.001. In my experience, such publications accurately identify the date of publication and are available online as of that date. EX1030 appears to be an authentic copy of Richard downloaded from <https://www.spiedigitallibrary.org/journalArticle/Download?urlId=10.1117%2F1.3522506>.

107. Richard discusses methods to “investigate the cellular mechanisms” of the brain cells of rats to assess “the beneficial effects of [lipoic acid] on the early cellular events that take place following the onset of ischemia.” EX1030.001-.002. Richard teaches performing single cell imaging by utilizing various standard

elements in optical microscopy, including “solenoid valves.” Richard discloses “using solenoid valves (Valvelink 8 controller, Automate Scientific, Berkeley, CA, USA)” for interchanging perfusion solutions into a “a laminar flow chamber (FCS2; Biopetechs, Butler, PA, USA).” *Id.* at .003.

J. Nikon (EX1043)

108. Nikon is a webpage entitled “Culture Chambers for Live-Cell Imaging” published by Nikon Instruments Inc. on its MicroscopyU platform—a source for microscopy information. As shown by the wayback machine, the Nikon webpage reproduced as EX1043 was publicly accessible by at least August 10, 2015, as stated at the top of the first page: “The Wayback Machine - <https://web.archive.org/web/20150810165805/http://www.microscopyu.com:80/articles/livecellimaging/culturechambers.html>.” I understand that webpages identified by the wayback machine are those that are distributed publicly, which is wholly consistent with this webpage distributed by Nikon Instruments, as Nikon Instruments would be expected to intend wide distribution for its commercial and educational purposes, and to successfully achieve this intended wide public distribution to those interested in the subject matter. This version of the webpage was downloaded December 27, 2023. EX1043.001 (see top left-hand corner).

EX1043 appears to be an authentic copy of Nikon downloaded from the website archive.²

109. Nikon provided an overview of live-cell imaging chambers. *See* EX1043.001. Specifically, Nikon described a variety of live-cell imaging chambers and their designs and components, including a multi-well chamber slide incubator shown in Figure 5(c) that includes an aspirator for fluid removal. *See id.* at .006 (Fig. 5C).

K. Skaft-Pedersen (EX1048)

110. Skaft-Pedersen is a scientific article published in 2012 (online December 13, 2011); Skaft-Pedersen et al., “A self-contained, programmable microfluidic cell culture system with real-time microscopy access,” *Biomedical Microdevices* 14: 385-399 (2012). EX1048.001. EX1048 appears to be an authentic copy of Skaft-Pedersen download from <https://link.springer.com/article/10.1007/s10544-011-9615-6>.

111. Skaft-Pedersen describes “a complete self-contained system for automated cell culture and experiments with real-time optical read-out” in

² The webpage address currently hosts essentially the same article with insubstantial differences, including a change in title and correction of typographical errors. *See* <https://web.archive.org/web/20150810165805/http://www.microscopyu.com:80/articles/livecellimaging/culturechambers.html>.

connection with biological assays. EX1048.001. In particular, Skafte-Pedersen discloses the use of a stepper motor for pump construction in the microfluidic system. *See id.* at .004.

L. Sands (EX1046)

112. Sands is a scientific article published in August, 2005; Sands et al., “Automated Imaging of Extended Tissue Volumes Using Confocal Microscopy,” *Microsc. Res. Tech.* 67: 227-239 (2005). EX1046.001. EX1046 appears to be an authentic copy of Sands downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/epdf/10.1002/jemt.20200?saml_referrer.

113. Sands describes a system for automated imaging of extended tissues using confocal microscopy to generate three dimensional images of tissue. EX1046.001, .005 (Fig. 3). To move the focal plane through the sample, the system utilizes a system with a linear motor to effectuate motion in the z-direction. *Id.* at .002.

M. Brooker (EX1040)

114. Brooker is a published U.S. patent application, US2011/0090562 A1, titled “Autofocus Apparatus,” published April 21, 2011. EX1040 at codes (10), (12), (43), (54).

115. Brooker discloses an autofocusing apparatus for a microscope. EX1040. To implement the autofocusing mechanism, Brooker explains that “position feedback is facilitated using, in one embodiment, linear encoder scales embedded within the stage construction.” *Id.* ¶ 0023.

N. Niman (EX1044)

116. Niman is a scientific paper published April 4, 2013; Niman et al., “Controlled microfluidic switching in arbitrary time-sequences with low drag,” *Lab Chip*, 13: 2389-2396 (2013). EX1044.001. In my experience, such publications accurately identify the date of publication. EX1044 appears to be an authentic copy of Niman downloaded from https://www.academia.edu/17007369/Controlled_microfluidic_switching_in_arbitrary_time_sequences_with_low_drag.

117. Niman addresses the “ability to test the response of cells and proteins to a changing biochemical environment” by using “total-internal reflection fluorescence microscopy to characterize the time-dependent change in concentration during switching of solutions near the device surface.” EX1044.001. As part of the experimental setup, Niman teaches the use of a Fluigent microfluidic control system, which was a commercially available control system used to control “pressure-driven flow and control of differential pressures to inlets and waste outlets” of the microfluidic system. *Id.* at .003.

O. Wessels (EX1050)

118. Wessels is a review article published in March, 2012; Wessels et al., “Light-Emitting Diodes in Modern Microscopy—From David to Goliath?” *Cytometry Part A*, 81A: 188-197 (2012). EX1050.001. In my experience, such publications accurately identify the date of publication. EX1050 appears to be an authentic copy of Wessels downloaded from <https://onlinelibrary.wiley.com/doi/epdf/10.1002/cyto.a.22023>.

119. Wessels teaches use of light-emitting diode (LED) illumination sources for fluorescence microscopy, and use of excitation filters to improve the properties of the light used for excitation of fluorophores in the sample being imaged. *Id.* at .005-.006, Fig. 1(A).

P. Winer (EX1051)

120. Winer is a scientific article published February 5, 2014; Winer et al., “Application of a three-dimensional (3D) particle tracking method to microfluidic particle focusing,” *Lab Chip* 14: 1443-1451 (2014). EX1051.001. In my experience, such publications accurately identify the date of publication. EX1051 appears to be an authentic copy of Winer.

121. Winer teaches a “three-dimensional (3D) particle tracking method” that was successfully able to determine “the 3D locations of cell-sized particles in

microscale flows.” *Id.* Winer uses the Fluigent control system to control the microfluidics of a device for fluorescent imaging of particles in 3D. *Id.* at .003.

X. CLAIM CONSTRUCTION

122. I do not believe that any express claim construction is required to determine that the ’554 Patent claims are invalid because the prior art teaches and/or discloses the claim limitations under any reasonable construction. My opinion that no express claim construction is required may change if the Patent Owner sets forth its own explicit claim constructions or construes claim limitations contrary to their reasonable construction, and I reserve the right to provide express claim construction(s) later in this proceeding in response.

123. I have been informed by counsel that in a parallel proceeding before a district court, the parties have not identified any terms that require construction in the ’554 Patent. Therefore, I do not expect any terms in the ’554 claims to be given any meaning other than their plain and ordinary meaning. My opinion may change if I am informed that the Court has construed any claim term in the ’554 Patent.

XI. PRIOR ART WOULD HAVE RENDERED OBVIOUS THE CLAIMS OF THE '554 PATENT

A. Ground 1: Claims 36-39, 43-44, 46-47, 54, 56, 59, 60, and 64-69 Would Have Been Rendered Obvious by the Combination of Zhuang, Cai, And Shen

1. Claim Element 36 [preamble]: A system comprising:

124. To the extent that the preamble is deemed limiting, it is my opinion that Zhuang discloses a system comprising a stage, a fluidics system, an optical system, a Z-axis motion control system and a computing system. EX1001 at Claim 36.

125. The elements of the claimed system can be broadly divided into two groups – an optical detection system (comprising a stage, a fluidics system, an optical system, a Z-axis motion control system, and a computer system) and software instructions associated with the computing system (comprising instructions to perform a method of detecting, identifying, and determining the location of nucleic acids). While I provide a brief overview of the system here, I discuss its components in greater detail further in my declaration under the respective claim limitations.

126. Zhuang's optical detection system can also be understood to comprise: (1) a stage; (2) a fluidics system; (3) an optical system, (4) a Z-axis motion control system, and (5) a computing system. Zhuang discloses a microscope stage configured to hold a sample. *See, e.g.*, EX1003.011 ("The sample was imaged on a home-built microscope constructed around an Olympus IX-71 body and a 1.45

NA, 100× oil immersion objective and configured for oblique incidence excitation.”).

127. Zhuang discloses a “fluidics system” composed of reservoirs holding fluid reagents and a fluid dispenser dispensing fluid from the reservoir to the sample with the help of a pump. *Id.* (“...and the flow through this chamber was controlled via a home-built fluidics system”).

128. Zhuang discloses an optical system comprising of an objective, light sources and detectors to receive light signals. *Id.* (“The sample was imaged on a home-built microscope.”). Zhuang discloses or teaches a Z-axis motion control system in disclosing that it allows spatial determination of nucleic acids in three dimensions. *Id.* at .012 (“For this purpose, we optically sectioned these cells by collecting stacks of images at different focal depths through the entire depth of the cells.”). Zhuang teaches the use of a three-dimensional stack in connection with its smFISH validation experiments, but this disclosure nonetheless confirms that Zhuang’s MERFISH system included a Z-axis control system because Zhuang makes clear that the same experimental setup was used both for MERFISH and smFISH. *Id.* (“The sample was then washed three times with 2×SSC and imaged in imaging buffer by using the same imaging geometry as described above for MERFISH imaging.”).

129. Zhuang further teaches employing a computing system in order to automate its methods of detecting, identifying, and determining the location of nucleic acids. *See id.* at .011 (“The entire system was fully automated, so that imaging and fluid handling were performed for the entire experiment without user intervention.”); *see also id.* (“Flow was then stopped, and then ~75 to 100 regions were exposed to ~25 mW 642-nm and 1 mW of 561-nm light and imaged.”).

130. According to Zhuang, this computing system is configured to perform a method of receiving images from the optical detection system described above, determining the optically-encoded signals from that system, and identifying the nucleic acid molecules and determining their three-dimensional positional information. For example, Zhuang teaches a “fully automated” system that allows imaging and fluid handling to be performed without any user intervention. *Id.*

131. Therefore it is my opinion, as set forth in greater detail below, that Zhuang discloses the system along with the recited components in Claim 36 of the ’554 Patent. Immediately below, I walk through where the prior art discloses each of the individual components of the claimed system.

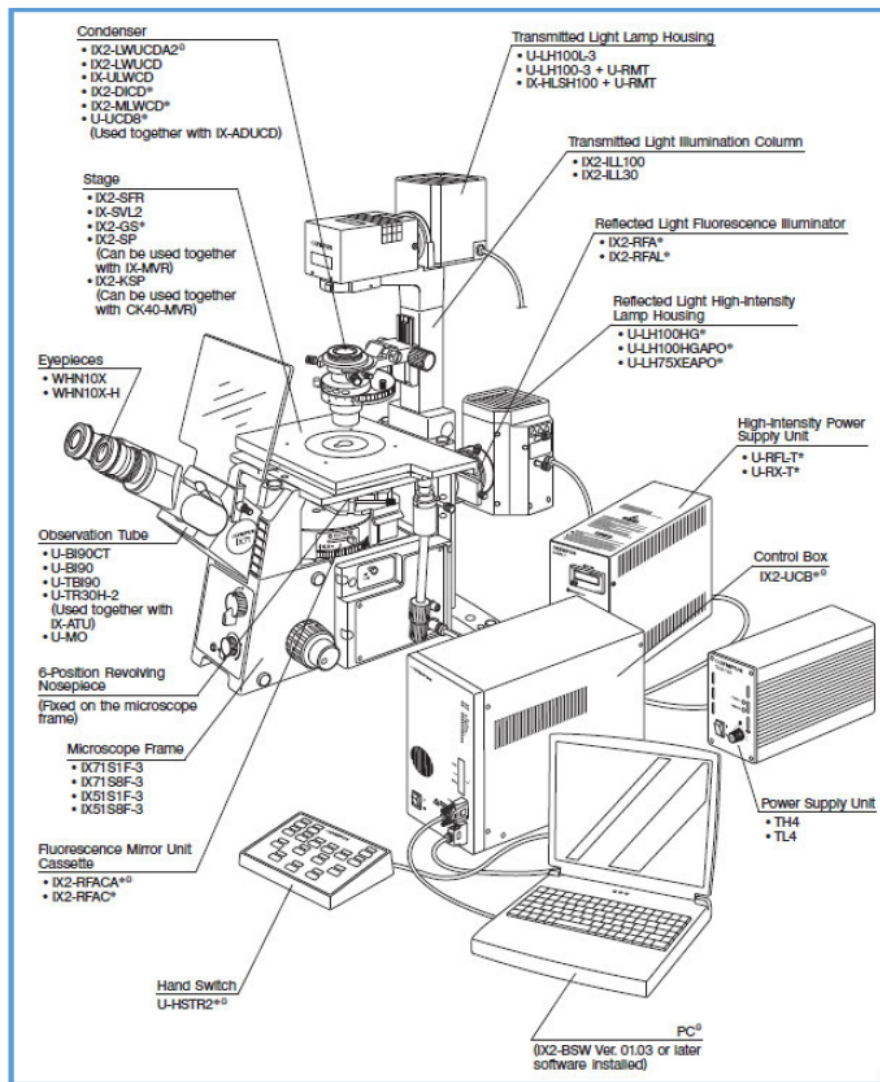
a. Claim Element 36[a]: a stage comprising:

132. It is my opinion that Zhuang discloses “a stage” as recited in Claim 36.

133. Zhuang teaches a sample imaged on a “home-built microscope constructed around an Olympus IX-71 body and a 1.45 NA, 100× oil immersion objective and configured for oblique incidence excitation.” EX1003.011.

134. This microscope system, like all microscope systems, includes a stage. For instance, the product literature regarding the Olympus IX-71 explains that “[m]otorised filter wheels, filter cube turret, condenser, light intensity control, shutters and *X/Y stage* offer a high level of flexibility for a broad range of automated tasks.” EX1033.010; *see also id.* (“High-precision stages controlled via joystick and/or PC.”), .019 (“Ceramic-coated coaxial stage with right-hand drive control, 50 mm (X) x 50 mm (Y) stroke, stage insert plate exchangeable (ø 110 mm).”).

135. Below is a schematic of the Olympus IX71 from its product manual:



EX1005.008. As the image shows, in the upper left, the system includes a stage where the sample can be placed for visualization. This stage is needed to act as a platform for the sample to be imaged using the optic system.

136. Starting immediately below, I walk through each of the components that the claim recites as being disclosed as part of the stage and explain where this is disclosed in the prior art.

(1) Claim Element 36[a][i]: a sample holder configured to receive a substrate comprising a sample having a volume;

137. It is my opinion that Zhuang discloses that the stage comprises a sample holder configured to receive a substrate comprising a sample having a volume.

138. Zhuang teaches the use of a “Biotech’s [sic] FCS2 flow chamber.” EX1003.011. Such a flow chamber was for the purpose of holding the sample. Specifically, this Biotech’s flow cell included within it a substrate in the form of a sample coverslip upon which sample cells were deposited. *See id.* (“Cells were plated on 22-mm, #1.5 coverslips (Biotech’s, 0420-0323-2)...); *id.* (“The sample coverslip was assembled into a Biotech’s [sic] FCS2 flow chamber...”).

139. Zhuang also discloses samples with a volume. Specifically, Zhuang used human primary fibroblasts, which are cells. *Id.* A POSA would have understood such cellular samples as having a volume. This is confirmed by the language of the ’554 Patent itself, which acknowledges that individual cells are a sample type that can be interrogated in 3D. EX1001 at 4:43-50, Fig. 1.

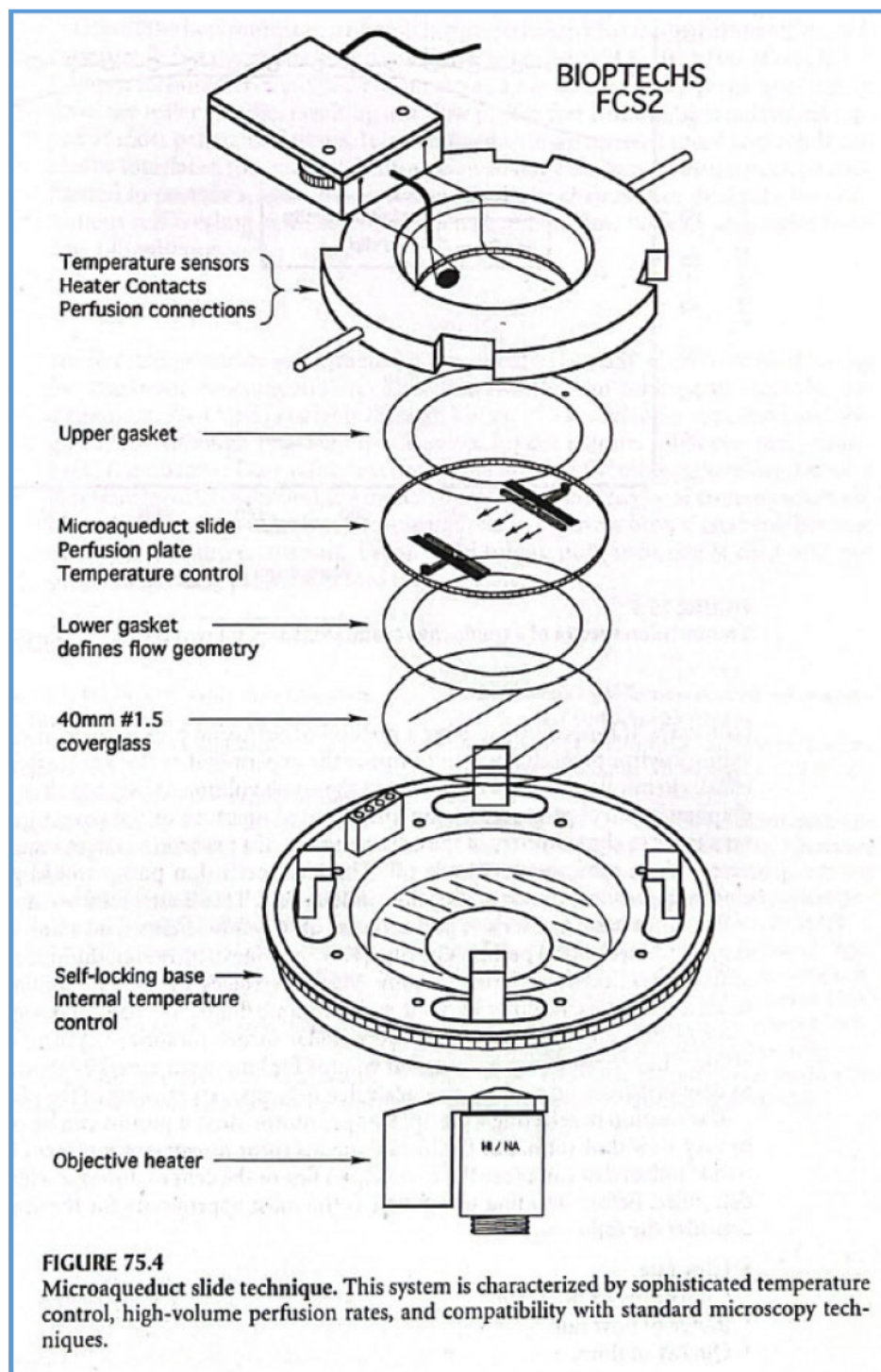
140. Zhuang also describes the use of “any optical sectioning technique” in MERFISH to allow “the imaging of RNAs in thicker cells or tissues.” EX1003.012. A POSA would have understood that “thicker” tissue samples imaged using an optical sectioning technique would refer to a sample having a 3D volume.

(2) Claim Element 36[a][ii]: a temperature control apparatus operatively coupled to said sample holder; and

141. It is my opinion that Zhuang discloses a temperature control apparatus operatively coupled to the sample holder.

142. Zhuang teaches the use of a Bioptechs FCS2 chamber, which would have been known to include a temperature control capability. EX1003.011. Long before the priority date of the '554 Patent (as early as 2009), the use of a temperature control elements with the flow chamber was well-established in the art. In fact, the Bioptechs FCS2 flow cell includes just such a system. *See, e.g.*, EX1007.004 (“The Bioptechs FCS2 chamber maintains the cells and media at 37°C. Unsheared (CT) cells were incubated for 120 min at 37°C....cells were subject to flow through the chamber to yield an average laminar shear stress of 15 dyn/cm² for 120 min at 37°C.”). A POSA would have further understood that various components provide and support maintenance of suitable temperatures, including temperature sensors, heating elements, and elements having thermal mass, i.e., having an ability to absorb, store, and release heat energy.

143. Further evidence supports that it was well-known to a POSA that the Bioptechs FCS2 flow cell includes temperature control. An expanded view of the FCS2 system with the conductive temperature sensors shown at the top is depicted in the figure below from a book chapter regarding the Bioptechs FCS2 flow cell:



(EX1015.017). As the image shows, the microaqueduct slide includes a “[t]emperature control” as does the self-locking base, which includes “[i]nternal temperature control.” *Id.*

144. Zhuang also discloses that the “objective was heated to 37°C with a Biopetechs objective heater.” EX1003.011. This is yet an additional temperature control. In my opinion, a POSA would have understood that this objective heater is operatively coupled to the sample holder as a POSA would have appreciated that it was the cells and perfused solutions that required the temperature to be maintained at 37°C. In particular, during an experiment using an oil immersion lens, the objective lens with the oil immersion medium droplet placed on the front lens element is moved to contact the coverslip with the attached sample on the opposite side of the coverslip. Thus, the heated objective is in thermal contact with the sample holder, i.e., the objective is transferring heat through the oil as a medium. The objective heater is therefore an additional temperature control system, additionally minimizing thermal gradients that would cause drift while imaging, and in the process of use becomes coupled to the stage and sample holder.

(3) Claim Element 36[a][iii]: An XY Motion Control System;

145. It is my opinion that Zhuang discloses an XY Motion Control System.

146. As described above, Zhuang teaches the use of an Olympus IX-71 microscope as the body for the homebuilt microscope. EX1003.011. This was a

system that included an X-Y motion control system, as set forth in the relevant documentation for the product. *See, e.g.* EX1005.022 (“To move the specimen to a desired position, rotate the X-axis knob (1) and Y-axis knob (2).”). Likewise, the 2009 product brochure for the system explains that the platform includes a “X/Y stage” that is a “[h]igh-precision stages controlled via joystick and/or PC.” EX1033.010; *see also id.* at .019 (“Ceramic-coated upper circular stage, 360° rotatable, 20 mm (X/Y) travel.”).

147. To the extent Patent Owner contends that the XY motion control system must be automated through motorization, this too is disclosed in Zhuang through its use of an Olympus IX-71 microscope. EX1003.011 (“The entire system was fully automated, so that imaging and fluid handling were performed for the entire experiment without user intervention...Flow was then stopped, and then ~75 to 100 regions were...[illuminated] and imaged.”). Also, evidencing what a POSA would have understood, the brochure for this system explains that “[m]otorisation is essential for full process automation. Consequently, all major microscope functions can be motorised – including focus, illumination, objective change and optical path selection.” EX1033.010. The brochure goes on to explain that the IX-71 system includes “[m]otorised filter wheels, filter cube turret, condenser, light intensity control, shutters and X/Y stage,” which “offer a high level of flexibility for a broad range of automated tasks.” *Id.*

148. Shen provides an example of the use of an automated X-Y motor control system as early as 2006, teaching that to carry out digital autofocus in a microscopy scan, one requires a “motorized x-y stage with linear encoding feedback.” EX1032.002. A POSA would have understood that linear encoding feedback refers to a feedback system that informs a computer on how to instruct the apparatus to move in the X-Y plane, and so automates X-Y motion.

149. Shen details its X-Y automation system, stating that the motorized stage uses a stepper motor with a “linear encoder of 100-nm resolution for each axis and is capable of attaching a piezo-electric transducer add-on for vertical travel of the specimen.” *Id.* at .004.

150. Thus, given the state of the art, it is my opinion that a POSA would have recognized that Zhuang, to perform MERFISH, would have used an automated system given that it built its microscope around the Olympus IX-71.

151. Moreover, it is also my opinion that, were it determined that Zhuang did not disclose a stage that includes an XY control system, a POSA would have found it obvious to include such an automated X-Y positioning system for the benefit of X-Y positioning of the sample being imaged with respect to the microscope objective. A POSA would have looked to resources such as Shen to find such automated systems.

152. A POSA would have been motivated to combine the teachings in Shen with the system described in Zhuang because Zhuang expressly suggests such a combination. Zhuang teaches the use of a “fully automated” system. EX1003.011. In my opinion, a POSA would have looked to resources such as Shen to find details of such a system and how to incorporate one into Zhuang’s system.

153. A POSA would have also had a reasonable expectation of success in combining the teachings. Shen teaches its automated system constructed onto an Olympus IX-71 microscope, which is exactly the microscope Zhuang uses. Shen also makes these modifications for the same goal, fluorescent imaging of a sample. 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 Zhuang.

b. Claim Element 36[b]: a fluidics system comprising:

154. It is my opinion that Zhuang discloses a fluidics system comprising of a reservoir configured to contain fluid reagents, a fluid dispenser and a pump configured to provide the fluid reagents from the reservoir to the sample holder.

155. As described above, Zhuang teaches that flow through the flow chamber is “controlled via a homebuilt *fluidics system* composed of three computer-controlled eight-way valves (Hamilton, MVP and HVXM 8-5) and a computer-controlled peristaltic pump (Rainin, Dynamax RP-1).” EX1003.011 (emphasis

added). This is a fully automated system and does not require input or control once programmed. *Id.*

156. Below, I further explain how Zhuang teaches all of the components that are claimed as being part of the “fluidics system.” To the extent that any of these elements are deemed missing from Zhuang, it would have been obvious to a POSA to have provided a reservoir, a fluid dispenser, and a pump, as detailed below.

(1) **Claim Element 36[b][i]: One Or More Reservoirs Configured To Contain One Or More Fluid Reagents; And**

157. It is my opinion that Zhuang discloses one or more reservoirs configured to contain one or more fluid reagents

158. Zhuang describes a number of fluid reagents washed over a sample as part of the MERFISH process, including liquid solutions of labeled readout probe, readout wash buffer, imaging buffer, and photobleaching buffer:

One milliliter of 10 nM of the appropriate fluorescently *labeled readout probe in readout hybridization buffer* (2×SSC; 10% v/v formamide, 10% w/v dextran sulfate, and 2 mM vanadyl ribonucleoside complex) was flown across the sample, flow was stopped, and the sample was incubated for 15 min. Then 2 mL of *readout wash buffer* (2×SSC, 20% v/v formamide, and 2 mM vanadyl ribonucleoside complex) was flown across the sample, flow was stopped, and the sample was incubated for 3 min. Two milliliters of *imaging buffer* comprising 2×SSC, 50 mM TrisHCl

pH 8, 10% w/v glucose, 2mMTrolox (Sigma-Aldrich, 238813), 0.5 mg/mL glucose oxidase (Sigma-Aldrich, G2133), and 40 mg/mL catalase (Sigma-Aldrich, C30) was flown across the sample (50). Flow was then stopped, and then ~75 to 100 regions were exposed to ~25 mW 642-nm and 1 mW of 561-nm light and imaged. Each region was 40 by 40 μm . The laser powers were measured at the microscope backport. Because the imaging buffer is sensitive to oxygen (51), the ~50 mL of imaging buffer used for a single experiment was made fresh at the beginning of the experiment and then stored under a layer of mineral oil throughout the measurement. Buffer stored in this fashion was stable for more than 24 hours. After imaging, the fluorescence of the readout probes was extinguished via photobleaching. The sample was washed with 2 mL of *photobleaching buffer* (2 $\times$ SSC and 2 mM vanadyl ribonucleoside complex), and each imaged region of the sample was exposed to 200 mW of 641-nm light for 3 s.

EX1003.011 (emphasis added). A POSA would have recognized that in an automated fluidics system as described in Zhuang, these fluidics reagents would have been in one or more reservoirs.

159. Zhuang also discloses that “the entire system was fully automated, so that imaging and *fluid handling* were performed for the entire experiment without user intervention.” *Id.* A POSA would have understood Zhuang’s automated system to necessarily comprise one or more reservoirs capable of holding fluid

reagents which are flowed to and from the sample for the purposes of hybridization, labelling, washing, imaging, and bleaching.

(2) Claim Element 36[b][iii]: 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

160. It is my opinion that Zhuang discloses a fluid dispenser operationally coupled to a pump that is configured to provide one or more fluid reagents from the reservoirs to the sample holder.

161. The system in Zhuang includes a “homebuilt fluidics system” which has a “computer-controlled peristaltic pump (Rainin, Dynamax RP-1).” EX1003.011. This Rainin Dynamax RP-1 peristaltic pump used in Zhuang is designed for transferring fluids. Indeed, Zhuang explicitly states that the fluidics system that includes this pump is for the purpose of controlling “flow through” the chamber FCS2 flow cell that holds the sample. *Id.* Therefore, there is a fluid dispenser coupled to the pump to provide fluid from the necessary reservoirs to the sample.

162. Additionally, given that Zhuang’s system is entirely automated, a POSA would have understood that the computer-controlled pump is operationally coupled to an automated fluid dispenser that dispenses the fluid stored in the reservoir to the pump. *Id.* (“the entire system was fully automated, so that imaging

and ***fluid handling*** were performed for the entire experiment without user intervention.”) (emphasis added). Accordingly, it is also my opinion that a POSA would have understood that the fluid from the reservoir is transferred from the reservoir to the sample holder via a fluid dispenser that is operationally coupled to the peristaltic pump.

c. Claim Element 36[c]: An Optical System Comprising:

163. It is my opinion that Zhuang discloses an optical system.

164. A POSA would have recognized the Olympus IX-71 inverted microscope used in Zhuang to have included an optical system in its use of an objective, light source, and cameras as detectors for receiving light signals. *See, e.g.,* EX1003.011 (“The sample was imaged on a home-built microscope constructed around an Olympus IX-71 body and a 1.45 NA, 100× oil immersion objective and configured for oblique incidence excitation.”). As I explain below, Zhuang further discloses all the elements that the claims identify as being part of the claimed “optical system.”

(1) Claim Element 36[c][i]: An Objective;

165. As I describe above, Zhuang discloses an optical system which comprises an objective. *See supra* Section XI.A.1.c.

166. Zhuang specifically discloses using a “100× oil immersion objective.” EX1003.011. This is part of the home-built microscope constructed around an

Olympus IX-71 body, which a POSA would have understood to have an objective, like all microscope systems. *See, e.g.*, EX1005.033-.034; EX1033.017, .019.

(2) Claim Element 36[c][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

167. It is my opinion that Zhuang discloses two or more light sources each configured to deliver light to the volume, where the two or more light sources are configured to emit two or more colors of light.

168. Zhuang teaches three light sources that illuminate the sample volume. For example, Zhuang discloses that “[i]llumination 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.” EX1003.011. A POSA would have understood from this disclosure that Zhuang 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 (*i.e.*, light at wavelength of 641, 561, and 405 nm).

169. Moreover, a POSA would have understood that providing light of different colors, *i.e.*, of different wavelengths, would typically entail using different

light sources. This is particularly the case when laser light sources are used, as a POSA would have known was the typical light source, because most lasers emit monochromatic light at discrete wavelengths.

170. Accordingly, in my opinion, a POSA would have understood Zhuang to teach using two or more light sources and, to the extent Zhuang might be deemed not to teach using two or more light sources, a POSA would have found it obvious to include two or more light sources (such as lasers) to provide two or more colors of light. Specifically, to the extent that the Board finds Zhuang does not sufficiently disclose two or more light sources configured to emit two or more colors of light, it is my opinion that it would have been obvious to modify Zhuang with Cai's teachings using multiple light sources for the benefit of obtaining greater diversity of signals for generating distinct barcodes.

171. Cai discloses a sequential barcoding method with probes labeled with different detectable moieties, such as fluorophores or colors, that are used in different cycles. EX1004 at 13:4-14:15. This sequential hybridization creates barcodes which allow for specific identification of mRNAs of interest. This is shown summarized in Cai both in the text and in Fig. 2:

Exemplary sequential barcoding of provided methods.

(a) Schematic of sequential barcoding. In each round of hybridization, multiple probes (e.g., 24) were hybridized on each transcript, imaged and then stripped by DNase I treatment. The

same probe sequences could be used in different rounds of hybridization, but *probes were coupled to different fluorophores*. (b) composite four-color FISH data from 3 rounds of hybridizations on multiple yeast cells. Twelve genes were encoded by 2 rounds of hybridization, with the third hybridization using the same probes as hybridization 1. The boxed regions were magnified in the bottom right corner of each image. The matching spots were shown and barcodes were extracted. Spots without co-localization, without the intention to be limited by theory, could be due to nonspecific binding of probes in the cell as well as mis-hybridization. The number of each barcode were quantified to provide the abundances of the corresponding transcripts in single cells. (c) Exemplary barcodes. mRNA 1: Yellow-Blue-Yellow; mRNA 2: Green-Purple-Green; mRNA 3: Purple-Blue-Purple; and mRNA 4: Blue-Purple-Blue.

Id. at 9:28-48

Fig. 2

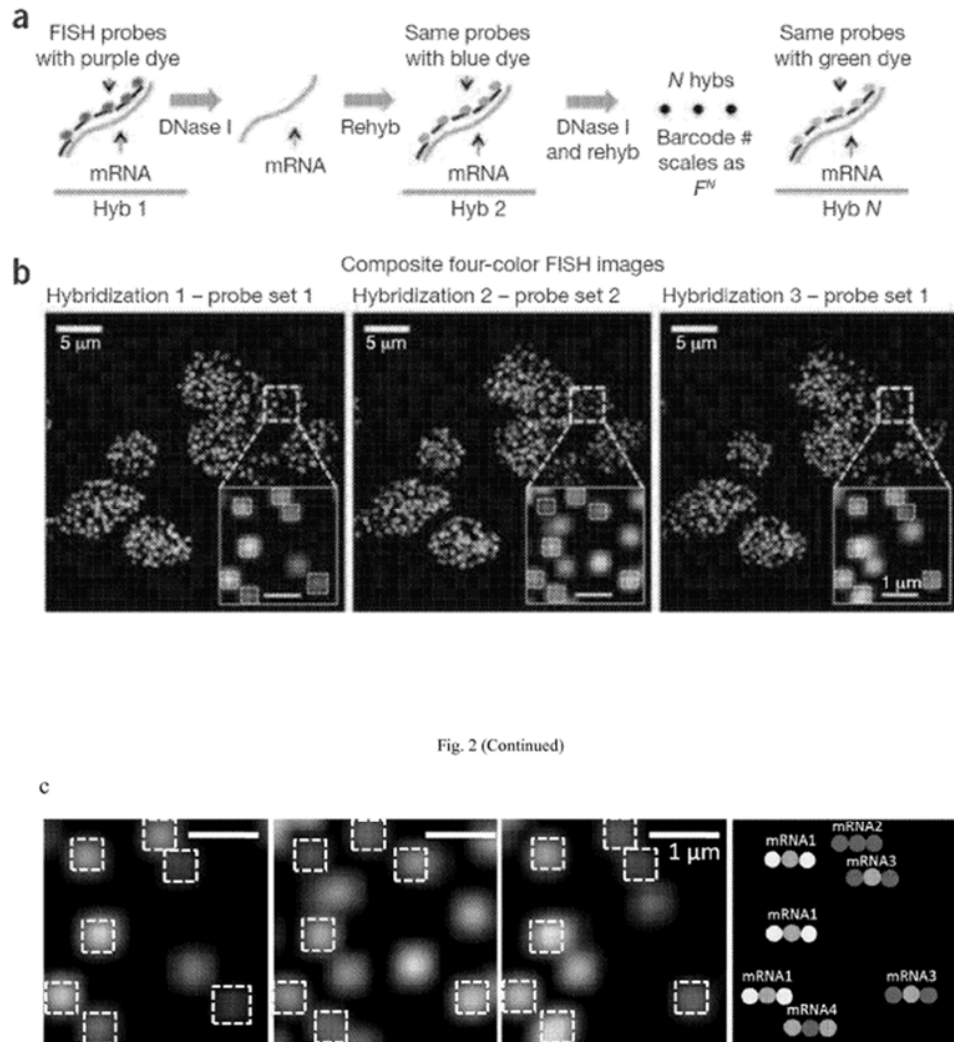


Fig. 2 (Continued)

Id. at Fig. 2.

172. In my opinion, the detection of different fluorophores as described in Cai would have required using different colors of excitation. This is particularly the case when laser light sources are used, as taught by Cai's listing of laser-excitabile dyes, because lasers emit monochromatic light. Accordingly, a POSA would have

understood Cai to teach using two or more light sources and, to the extent it might be deemed not to, a POSA would have found it obvious to include two or more light sources, such as lasers, to provide two or more colors of light for the benefit of greater diversity of signals for generating distinct barcodes given that its use was standard prior to 2015.

173. A POSA would have been motivated to combine the teachings of multiple colors in Cai with Zhuang's teachings because they would have understood that increasing the number of excitation light sources providing different colors of light would provide increased flexibility as to generating a greater number of distinct signals in each hybridization and imaging cycle.

174. Generating a greater number of distinct signals in each cycle, a POSA would also have understood, would provide the benefits of obtaining the same amount of information in less time and with better preserved sample, i.e., a sample that has been subjected to less process that damages it, than could be obtained using a single light source providing a single color of light.

175. In fact, Zhuang teaches precisely this, stating that "[a]lthough an increase in the number of bits by adding more hybridization rounds would increase the data collection time and potentially lead to sample degradation, these problems could be mitigated by utilizing multiple colors to readout multiple bits in each round of hybridization." EX1003.008. Accordingly, it is my further opinion that even if

Zhuang had not expressly taught using two or more light sources to deliver two or more colors of light to the sample volume, it would have been obvious to a POSA to have done so with a reasonable expectation of success. Indeed, such an approach had previously been successfully used in Cai, as reflected in the real-world data shown in Figure 2 of Cai above.

(3) Claim Element 36[c][iii]: one or more detectors configured to receive light signals from said volume;

176. It is my opinion that Zhuang discloses one or more detectors configured to receive light signals from the volume.

177. As I discuss above, Zhuang teaches an optical system which includes one or more detectors configured to receive light from the sample. Specifically, Zhuang teaches an EMCCD camera to receive light signals from the sample volume:

Fluorescence was separated with a QuadView (Photometrics) by using the dichroics T560lpxr, T650lpxr, and 750dcxxr (Chroma) and the emission filters ET525/50m, WT59550m-2f, ET700/75m, and HQ770lp (Chroma) and imaged with an EMCCD camera (Andor, iXon-897). The camera was configured so that a pixel corresponds to 167 nm in the sample plane.

EX1003.011.

178. It is my opinion that a POSA would recognize an EMCCD camera as a detector configured to receive light signals from the volume. Thus, it is my opinion

that Zhuang discloses one or more detectors configured to receive light signals from said volume.

d. Claim Element 36[d]: a Z-axis motion control system configured for Z-motion of an objective focal plane through said volume; and

179. It is my opinion that Zhuang teaches a Z-axis motion control system configured for Z-motion of an objective focal plane through said volume.

180. As I've described, Zhuang teaches the use of a homebuilt microscope around an Olympus IX-71 body. A POSA would have known that the Olympus IX-71 microscope, like all microscopes, included an up-down control system for focusing, which allows the objective to be moved up and down and moves the focal plane through the sample volume. EX1005.019-.020 ("The stage up/down movement using the fine adjustment knob is not limited."); *see also id.* at .055 ("Focusing up/down stroke: 9 mm (7 mm above focusing position plus 2 mm below focusing position.>"). This includes, for instance, a fine adjustment knob, which would permit a scientist to fine tune the focus and move the focal plane to precisely what portion of the volume they want to observe. *Id.* at .019-.020, .055.

181. Likewise, the product brochure also evidences what a POSA would have understood to be well-known in the art as to Z-motion control systems, as it includes specifications stating that the system includes "[v]ertical stage movement: 9 mm stroke with upper limit stopper, coaxial coarse and fine focusing knobs,"

where a POSA would have understood focusing to require Z-axis movement of the objective. EX1033.019. The brochure likewise teaches that “[a]ll series of acquisitions are saved together with all experimental parameters in a multidimensional image format. This means that a single image file can consist of multiple images taken at e.g. different Z-position....” *Id.* at .012. Referring to different “Z-position,” the product documentation for the system used in Zhuang makes clear that the system included a Z-axis motion control system for moving the focal plane through the sample.

182. Further, Zhuang teaches that “[c]onstant focus was maintained throughout the imaging process with a home-built, autofocusing system.” EX1003.011. It is my opinion that a POSA would have known that to maintain constant focus, the existing focus system from the microscope body would have been outfitted with a motorized or piezo Z-axis motion control system to permit automated movement of the focal plane through the sample. Indeed, the only way constant focus could possibly be maintained is if there was a mechanism to move the objective focal plane through the sample.

183. To the extent Patent Owner contends that the claims require motorized automation of the Z-axis motion control system and that Zhuang does not expressly teach this, automation by motorization would have been obvious in view of Zhuang alone. Zhuang discloses or teaches a Z-axis motion control system in disclosing

that it allows spatial determination of nucleic acids in three dimensions in connection with its smFISH validation studies. *Id.* at .012 (“For this purpose, we optically sectioned these cells by collecting stacks of images at different focal depths through the entire depth of the cells.”). While Zhuang teaches the use of a three-dimensional stack in connection with its smFISH validation experiments, this nonetheless confirms that Zhuang’s MERFISH system included a Z-axis control system because Zhuang makes clear that the same experimental setup was used both for MERFISH and smFISH. *See id.* (“The sample was then washed three times with 2×SSC and imaged in imaging buffer by using the same imaging geometry as described above for MERFISH imaging.”).

184. A POSA would have known that the Olympus IX-71 class of microscopes were routinely automated for Z-axis motion control well before 2015, including through the use of supplemental components. The 2009 brochure for these instruments explains, for instance, that “[m]otorisation is essential for full process automation. Consequently, all major microscope functions can be motorised – including focus, illumination, objective change and optical path selection. In addition, the Olympus modular motorization allows you to choose a level of automation that perfectly matches your requirements.” EX1033.010. The sister model to the Olympus IX-71 microscope included a “[h]igh-precision internal Z-motor” that had a “10 nm step size and 1 µm reproducibility independent of

movement direction. Fine and coarse movement with 3 mm/sec. maximum speed.”

Id.

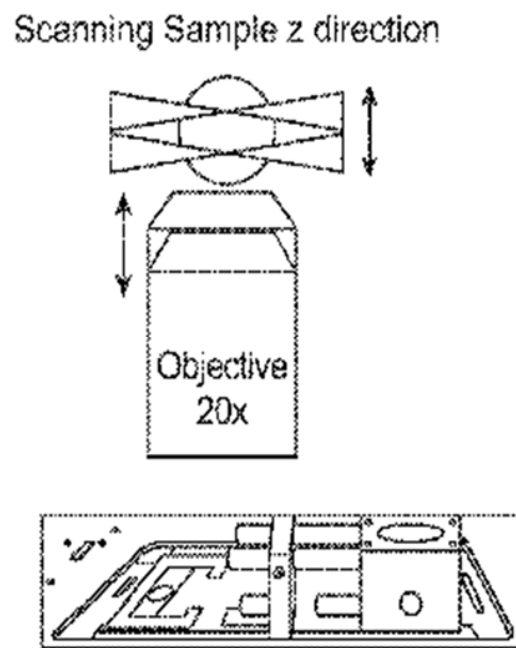
185. Shen, again, provides an example for this kind of automation in the Olympus IX-71 microscope. In fact, Shen acknowledges that as of 2006, “various solutions [were] possible” for “micropositioning the objective lens in its axial, or z position.” EX1032.004. Shen used a piezo-electric transducer to accomplish motorized axial, or Z-axis, focusing. *Id.* Other options mentioned, however, include a product known as PIFOC, a piezo-stage insert, an electric stepper motor with linear encoded feedback, or a combination of these options. *Id.* These were commonplace technologies for this kind of automation. Thus, the capabilities of automating the Z-axis focus in the very microscope Zhuang uses were well-established by no later than 2006.

186. In my opinion, a POSA would have been motivated to combine the teachings in Shen with Zhuang’s system because Zhuang suggests such a combination. Zhuang teaches the use of an autofocus system. EX1003.011. It is my opinion that a POSA interested in MERFISH would have looked to sources such as Shen to determine specific ways to build an autofocus system in an Olympus IX-71 microscope.

187. Further, it is my opinion that a POSA would have had a reasonable expectation of success in combining the teachings from Shen with Zhuang. Shen

uses the same microscope, the Olympus IX-71, with which Zhuang built the MERFISH system. Thus, a POSA would have reasonably expected the same type of modifications to be successful.

188. Cai similarly teaches automation that includes a Z-axis control system. Specifically, Cai, in the rightmost panel of Figure 17, depicts an example of a Z-axis motion control system. In this panel, the “objective is moving synchronically with the illumination sheet, *allowing scanning the sample along z-axis.*” EX1004 at 47:42-45 (emphasis added).



Id. at Fig. 17 (rightmost panel). Notably, the microscope that appears in Fig. 17 of Cai is the Olympus IX-81, which is the sister product the Olympus IX-71

microscope, which had been available well before 2015 and is discussed in the brochure for the Olympus IX-71. *See* EX1033.

189. A POSA would have been motivated to combine the relevant teachings in Cai with Zhuang. Zhuang describes its goal as determining “[k]nowledge of the expression profile and spatial landscape of the transcriptome in individual cells....” EX1003.002 (Abstract); *see also id.* at .005 (“As an imaging-based approach, MERFISH also allowed us to investigate the spatial distributions of many RNA species simultaneously.”). In fact, Zhuang expressly states that “because a fraction of the cell could be outside the imaging area or focal depth” that “*[t]iling* images of adjacent areas and *adjacent focal planes* could be used to improve the counting accuracy.” *Id.* at .012 (emphasis added). It is my opinion that to accomplish this sort of tiling and to understand the spatial landscape of the transcriptome in three dimensions, a POSA would have known that a Z-axis motion control for the focal plane would have been required. *See, e.g.,* EX1019 (describing a myriad of technologies available for three-dimensional microscopy as of 2011).

190. Further, such Z-axis motion controls were standard in the art and well known to a person of ordinary skill. The ’554 Patent describes nothing inventive about the Z-axis control, the specification stating only that “[a] Z-axis linear motor with linear encoder feedback for Z motion and Z scanning of objective focal plane through a sample is provided.” EX1001 at 27:13-15. The Examiner recognized

as much in the Notice of Allowance, where he wrote the primary prior art reference that had formed the basis for a rejection teaches a “microscope system for imaging a live biological specimen comprising a specimen chamber, a specimen holder, a perfusion system, *a microscope system for configured for performing volumetric imaging*, and an optical scanner device.” EX1036.006 (emphasis added).

191. That Z-axis motion control systems were standard and well-known is also confirmed by Shen, Cai, and other references dating before 2015. As discussed above, Shen teaches “various solutions” for “micropositioning the objective lens in its axial, or z position” were known by 2006 and Cai teaches the use of three-dimensional scanning along the Z-axis, and that this is to provide “three-dimensional spatial information of a target.” EX1032.004; EX1004 at 21:50-51. Further, Larsson et al. in 2010 reported on the collection of images as “z-stacks” in a FISH protocol. EX1008.005 (Online Methods). The long-known success of these methods would have provided a POSA with a reasonable expectation that the application of Z-axis scanning and imaging to Zhuang’s MERFISH protocol would have been successful.

192. Thus, it is my opinion that a POSA would have understood from Zhuang disclosure of methods for imaging in three-dimensions that Zhuang’s apparatus included a Z motion control system for Z-motion of the objective focal plane through the sample volume. It is further my opinion, that to the extent the

Board finds that Zhuang does not sufficiently disclose a Z-motion control system, it would have been obvious to combine the teachings of a Z-motion control system from Shen and/or Cai.

- e. **Claim Element 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:**

193. Zhuang discloses a computing system including a computer readable storage medium having program instructions that are executable by a processor of the computing system to cause the processor to perform a method of receiving a plurality of images, determining a plurality of optically-encoded signals, identifying one or more nucleic acid molecules, and determining three-dimensional positional information of the one or more nucleic acid molecules.

194. Zhuang teaches a “fully automated” system, where “imaging and fluid handling were performed for the entire experiment without user intervention. EX1003.011. The fluidics system described was composed of “three *computer-controlled* eight-way valves (Hamilton, MVP and HVXM 8-5) and a *computer-controlled* peristaltic pump....” *Id.* (emphasis added). In my opinion, a POSA would have understood that to achieve such automation, a computer system would

have been used. The computer system would have contained instructions to permit the system to operate without human intervention once it started.

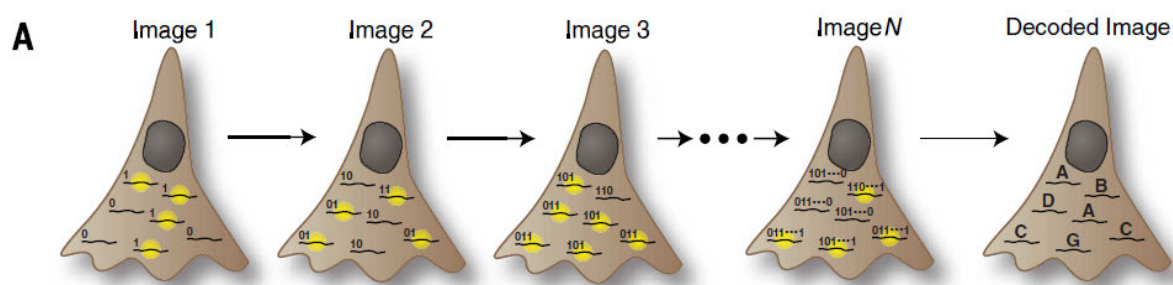
195. Further, as I describe below, it is my opinion that the computer program instructions used in automation would include the instructions that are recited in the claim.

- (1) **Claim Element 36[e][i]: 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;**

196. It is my opinion that Zhuang discloses this claim element.

197. Zhuang teaches generating images from a plurality of read-out cycles and analysis of the images using a computer, which requires the computer to receive the images. EX1003.011. Zhuang generally describes MERFISH as a “single-molecule imaging approach that allows the copy numbers and spatial localizations of thousands of RNA species to be determined in single cells.” *Id.* at .002 (Abstract).

198. Figure 1A demonstrates a plurality images from a plurality of readout cycles, wherein the plurality of images comprises a plurality of light signals. In this figure, each RNA species (the small, black lines in the cell) is coded with an N-bit binary word. *Id.* at .003. A “binary word” refers to the presence or absence of fluorescence. In other words, the scientist determines a threshold at which fluorescence is detected. At any spot wherein the threshold is met, a value of “1” is assigned. Where the threshold is not met, a value of “0” is assigned. *Id.* at .012.

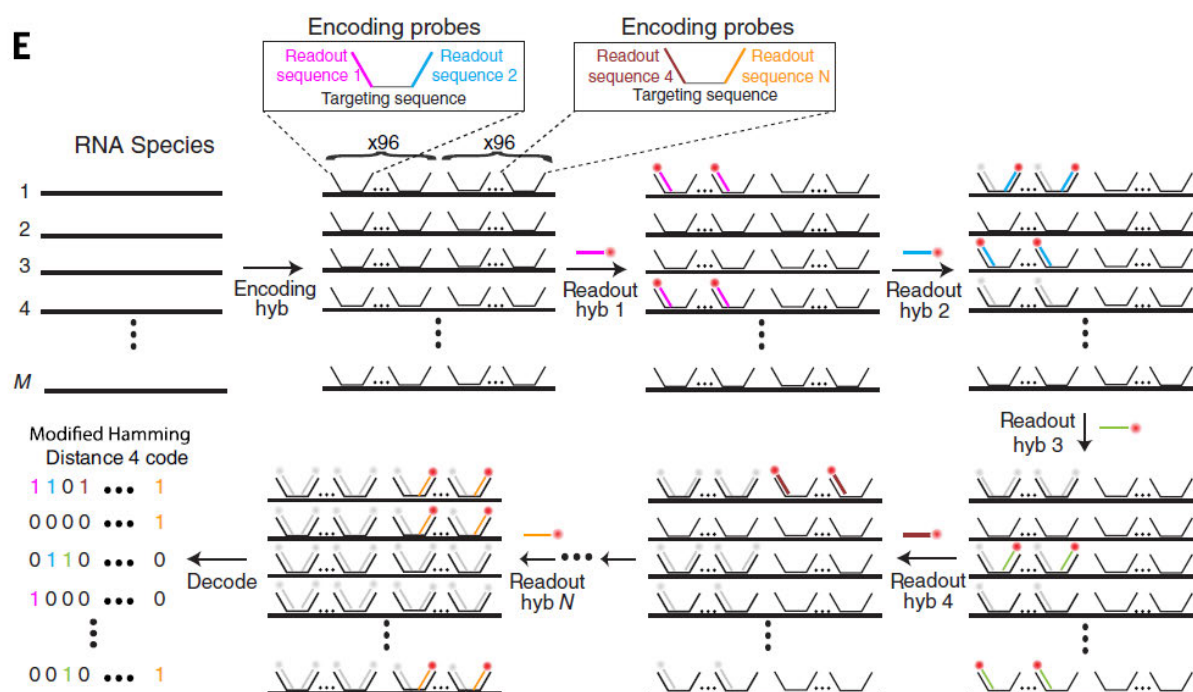


Id. at Fig. 1(A).

199. As shown in Figure 1A, this binary word process can be repeated over numerous images, up to N times, where N just represents the number of images. After N images are received, each unique RNA would have a “code word” assigned to it based on the string of binary “letters,” *i.e.*, a series of 1s and 0s marking which rounds hybridized and didn’t. These words are then decoded by using a preset decoding matrix, which determines if a measured binary word matches a code word of a specific RNA. *Id.* at .012.

200. Each yellow spot in this figure is a light signal associated with a fluorescent labeled probe hybridized to a readout nucleic acid sequence of an encoding probe that is hybridized to a nucleic acid of interest. *Id.* at .003-.004.

201. Figure 1E further illustrates this process. In this Figure, each RNA species is labeled with “encoding probes that convert the RNA into a specific combination of readout sequences.” *Id.* at .003. Each of these encoding probes contains a “specific combination of readout sequences,” which through repeated rounds of hybridization and detection form a barcode identifying the RNA species.



Id. at Fig. 1(E).

202. As Figure 1E showcases, once the targeting sequence is able to hybridize to a RNA molecule (shown as black lines 1-M), each round of

hybridization has probes hybridizing only to particular loci based on the targeting probe and the encoding probes attached. A unique readout probe is introduced each round, and the detection of the light signal occurs only when there is hybridization. *Id.* at .003-.004, .012.

203. Further, it is my opinion that Zhuang discloses that the light signals it measures have a 3D spatial position with the sample volume. Because the light signal is detected *in situ* only when the readout probe is hybridized, a POSA would recognize that the position of the light signals corresponds to the spatial position of nucleic acid molecules.

204. This spatial position can be a 3D position found within the volume. Zhuang 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.” *Id.* at .012. Thus, it is my opinion that a POSA in possession of Zhuang’s disclosures would recognize that MERFISH has the capability for three-dimensional spatial positioning.

205. To the extent Zhuang is deemed not to disclose this kind of three-dimensional imaging, it is my opinion that Cai discloses it and it would have been obvious to modify Zhuang in view of Cai to include this feature.

206. As I describe above, Cai teaches using seqFISH to profile nucleic acid molecules and obtain “three-dimensional spatial information.” EX1004 at 21:50-

51. Cai teaches the claimed Z-axis motion control system to permit three-dimensional interrogation through a stack of focal planes. *See supra* Section XI.A.1.d.

207. As Zhuang has the goal of identifying “distinct spatial distribution patterns of RNAs,” 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. They likewise would have recognized that 3D single molecule approaches were well-known in the art, as evidenced by the prior art, such as Zessin. *See generally* EX1052 (and references cited therein).

208. Further, it is my opinion that a POSA would have had a reasonable expectation of success in combining the references. Three-dimensional FISH was routine and well-known long before the 2015 priority date. As early as 2003, 3D optical sectioning and 3D interactive analysis software was used in conjunction with FISH to visualize mRNA *in situ*. EX1010.001-.002. Larsson et al., in 2010, collected images as Z-stacks to permit three-dimensional multiplexed analysis using FISH. EX1008.003. And Perillo et al. enhanced the 3D localization of individual RNA transcripts using a microscope for Z-localization. EX1011.002 (Abstract). MERFISH was an extension of existing FISH technologies that used sequential hybridization and error correction to increase multiplexity. It is my opinion, and a

POSA would have known, that this extension does not limit 3D detection and may even aid it.

- (2) **Claim Element 36[e][ii]: 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;**

209. It is my opinion that Zhuang discloses this claim element.

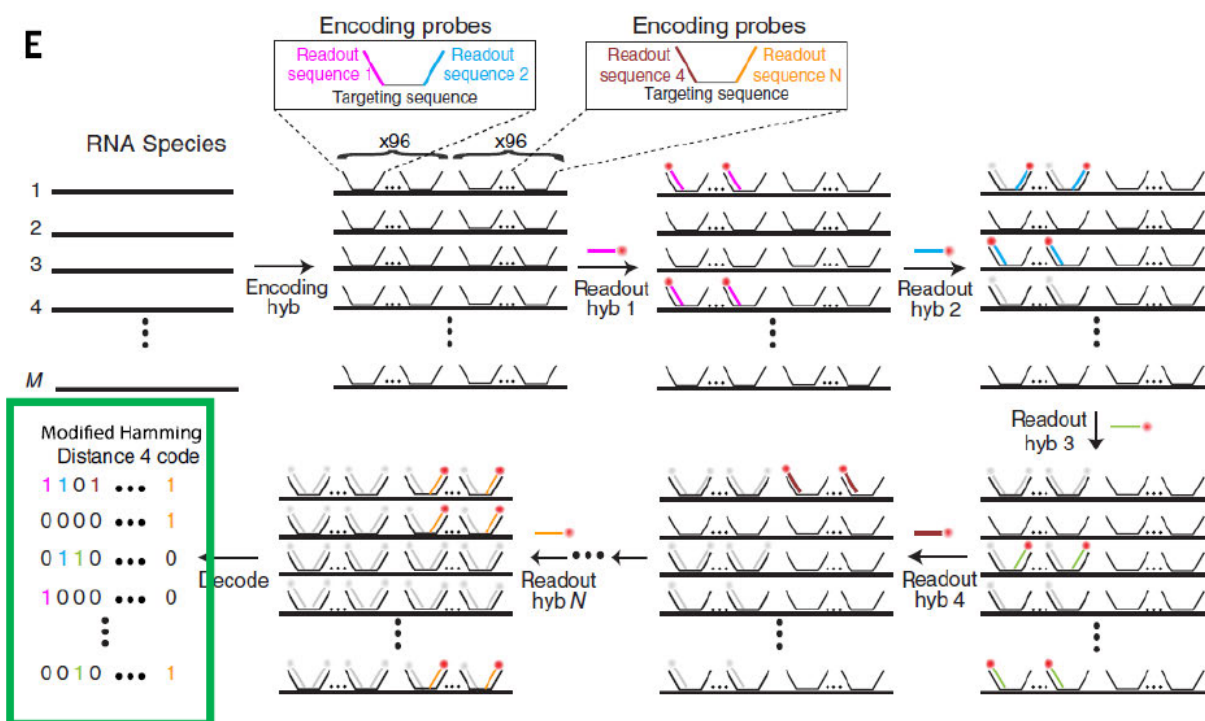
210. Zhuang teaches “determining a plurality of optically-encoded signals from said plurality of light signals obtained from said plurality of read-out cycles.” As I discuss above, Zhuang teaches generating a plurality of light signals from said plurality of read-out cycles and having a computer receive these signals. *See supra* Section XI.A.1.e.(1) (describing how plurality of images from a plurality of read-out cycles comprises a plurality of light signals). Further, Zhuang teaches that a computer can determine optically-encoded signals, such as discussed above in relation to Figure 1, by correlating the light used to excite a sample with the images acquired of the sample containing the various signaling entities:

Fluorescent spots were identified and localized in each image by using a multi-Gaussian-fitting algorithm assuming a Gaussian with a uniform width of 167 nm. This algorithm was used to allow

partially overlapping spots to be distinguished and individually fit. RNA spots were distinguished from background signal—signal arising from probes bound nonspecifically, by setting the intensity threshold required to fit a spot with this software.

EX1003.012; *see also supra* Section XI.A.1.e.(1) (discussing Zhuang, Fig. 1(E)).

211. Zhuang explicitly teaches determination of optically-encoded signals from light signals obtained over a plurality of read-out cycles. *See* EX1003 at Fig. 1(E) (discussed above). As I explain above, Fig. 1(E) illustrates multiple read-out cycles containing multiple rounds of hybridization of the RNA species with fluorescent readout probes. These data from the multiple read-out cycles are used to generate optically-encoded signals, as highlighted below (green box).



Id. at Fig. 1(E) (annotations added). These optically-encoded signals permit interpretation of the barcode generated by readout sequence, and so can be used to identify the RNA species.

212. Zhuang would have suggested to a POSA “wherein said plurality of optically-encoded signals comprises said two or more colors of light detected by said one or more detectors in series.” As I discuss above, Zhuang teaches two or more light sources that emit two or more colors and using them to activate light emitting entities, including light sources that activate the fluorophores of fluorophore-labeled probes. *See supra* Section XI.A.1.c.(2). Also, as discussed above, it would have been obvious to a POSA from this disclosure that multiple light sources could be used to excite the signaling entities in the sample to emit two or more colors of light, generating two or more light signals that are detected in the series of hybridization and imaging rounds, also discussed above, and that these two or more colors of light detected by one or more detectors in series would be reflected in the optically-encoded signals.

213. Further, to the extent that Zhuang is not found to suggest providing a plurality of optically-encoded signals comprising two or more colors, it would have been obvious to modify Zhuang in view of Cai to arrive at the use of multiple colors for benefit of generating greater diversity and number of distinct signals in each hybridization and imaging cycle. *See supra* Section XI.A.1.c.(2).

(3) Claim Element 36[e][iii]: identifying said one or more nucleic acid molecules based on said plurality of optically-encoded signals; and

214. It is my opinion that Zhuang discloses identifying one or more nucleic acid molecules based on the plurality of optically-encoded signals.

215. As I describe above, Zhuang describes the use of a software algorithm to decode the barcode based on the identification fluorescent spots. EX1003.012 (“Fluorescence spots in different hybridization rounds were connected into a single string, corresponding to a potential RNA molecule, if the distance between spots was smaller than 1 pixel....Measured words were then decoded into RNA species using the 16-bit MHD4 code or the 14-bit MHD2 code.”) Moreover, a POSA would have found it obvious to use additional or different probes as necessary for the optically-encoded signals to identify particular nucleic acids molecules. *See supra* Section XI.A.1.e.(1)-(2).

(4) Claim Element 36[e][iv]: determining three-dimensional positional information of said one or more nucleic acid molecules.

216. It is my opinion that Zhuang discloses determining three-dimensional positional information of the one or more nucleic acid molecules.

217. Zhuang discloses a computing system configured to perform a method to determine three-dimensional positional information of nucleic acid molecules by expressly teaching automating the disclosed methods which includes processing of

data to obtain three-dimensional spatial information for one or more nucleic acid molecules. EX1003.012. Moreover, it is my opinion that it would have been obvious to a POSA that Zhuang's automated system be configured to perform this method to determine three-dimensional positional information of one or more nucleic acid molecules.

218. In my opinion, a POSA would have understood Zhuang to disclose and teach determining three-dimensional positional information of one or more of the nucleic acid molecules it identifies based on its disclosures of an automated system capable of imaging and analyzing the images to develop a three-dimensional representation of the locations of nucleic acid molecules. *See also supra* Section XI.A.1.e.(1).

219. Further, to the extent Zhuang 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 Zhuang to permit a POSA to use MERFISH to determine three-dimensional positional information about one or more nucleic acid molecules. EX1004 at 21:50-51; *see supra* Section XI.A.1.e.(1).

2. Claim 37: The system of claim 36, wherein said substrate comprises a flow cell.

220. It is my opinion that Zhuang discloses a system of claim 36 wherein the substrate comprises a flow cell.

221. As I discuss above, Zhuang discloses or renders obvious a system including a sample holder that is configured to receive a substrate that comprises a sample with a volume. *See supra* Section XI.A.1.a.(1). Specifically, Zhuang identifies a “Bioptech’s FCS2 flow chamber” capable of holding the sample. EX1003.011 (“sample coverslip was assembled into a Bioptech’s FCS2 flow chamber”). Zhuang further discloses that “the flow through this chamber [is] controlled via a home-built fluidics system.” *Id.* This fluidics system, as discussed above in Section XI.A.1.b., allows the transfer of fluid to and from the flow chamber. In my opinion, from these teachings, a POSA would have understood Zhuang’s flow chamber (*i.e.* the sample holder) which is configured to receive a substrate to also comprise a flow cell. In particular, a POSA performing Zhuang’s method for live cell imaging would find it useful to utilize a flow cell for continuous flow of samples over a specified area for observation.

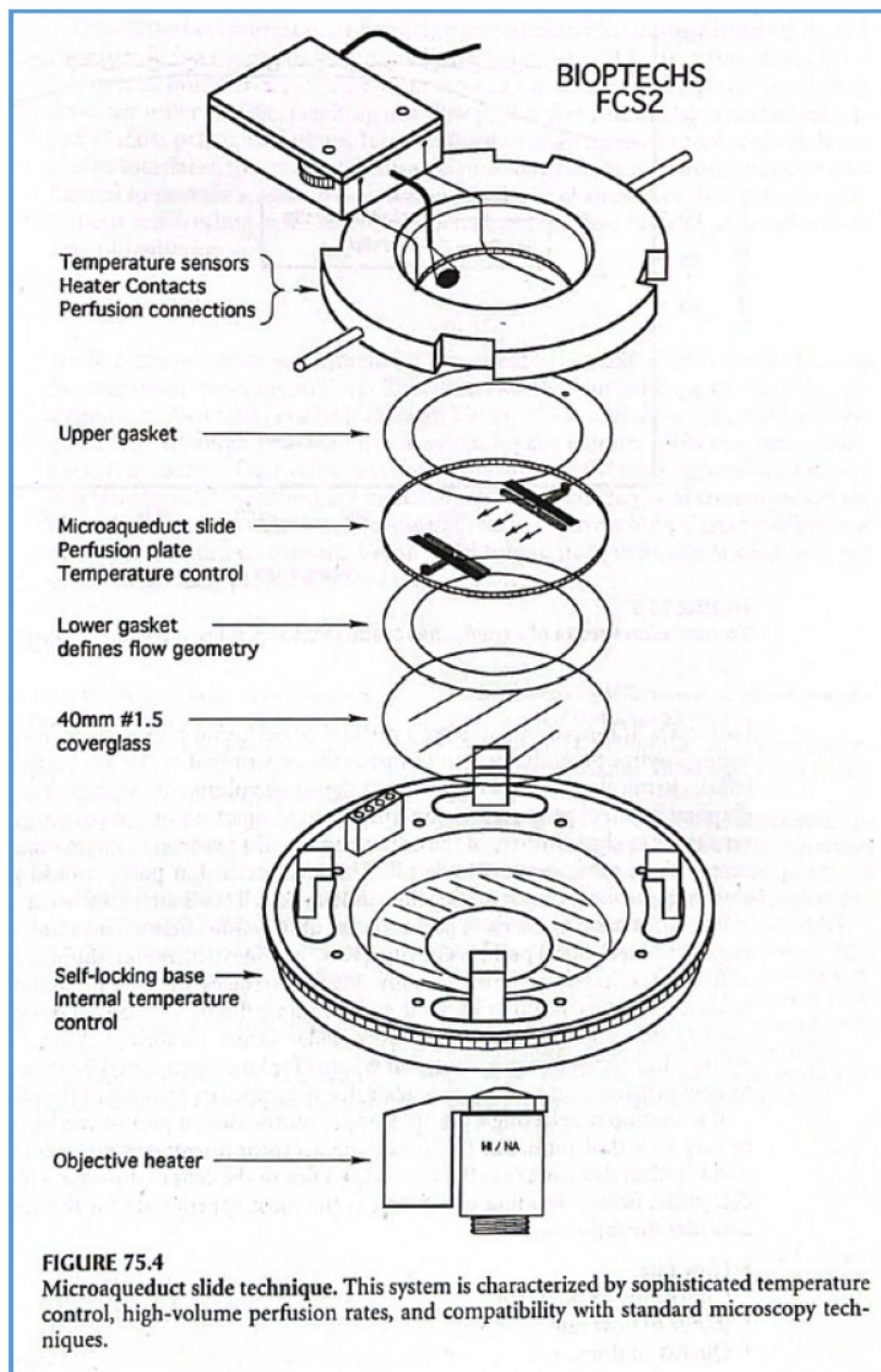
3. Claim 38: The system of claim 37, wherein said flow cell is a closed flow cell.

222. It is my opinion that Zhuang discloses a system of claim 37 where the flow cell is a closed flow cell.

223. As I describe above in Section XI.A.2, Zhuang teaches the “Bioptechs FCS2 flow chamber,” comprises a flow cell. It is my opinion that a POSA would have understood the flow cell to be closed flow cell, as publications from prior 2015 taught. *See, e.g.*, EX1009.012 (“The experimental details described here are for

the FCS2 closed perfusion system (Bioptechs, Inc.), but may apply to the use of other systems as well. A closed chamber system enables the acquisition of data from cells in optimal viability conditions, and the perfusion of fresh media will help minimize photodamage.”).

224. An expanded view of the FCS2 flow cell system is shown below, and helps make clear that the FCS2 flow cell is a closed flow cell:



EX1015.017. This image shows the different components that are stacked together to assemble the flow cell. As the image above shows, there is a “microaqueduct

slide” that is separated from a “coverglass” by a “lower gasket that defines flow geometry.” The “coverglass” on top of the microaqueduct slide leads to a closure of the flow cell. Thus, it is my opinion that a POSA would have understood Zhuang to be teaching a system with a closed flow cell.

4. Claim 39: The system of claim 38, wherein said closed flow cell comprises a fluid inlet and a fluid outlet, and wherein said fluid inlet is fluidically coupled to said fluid dispenser.

225. It is my opinion that Zhuang discloses and renders obvious a system of claim 38 where the flow cell is a closed flow cell that comprises a fluid inlet and fluid outlet, and where the fluid inlet is fluidically coupled to the fluid dispenser.

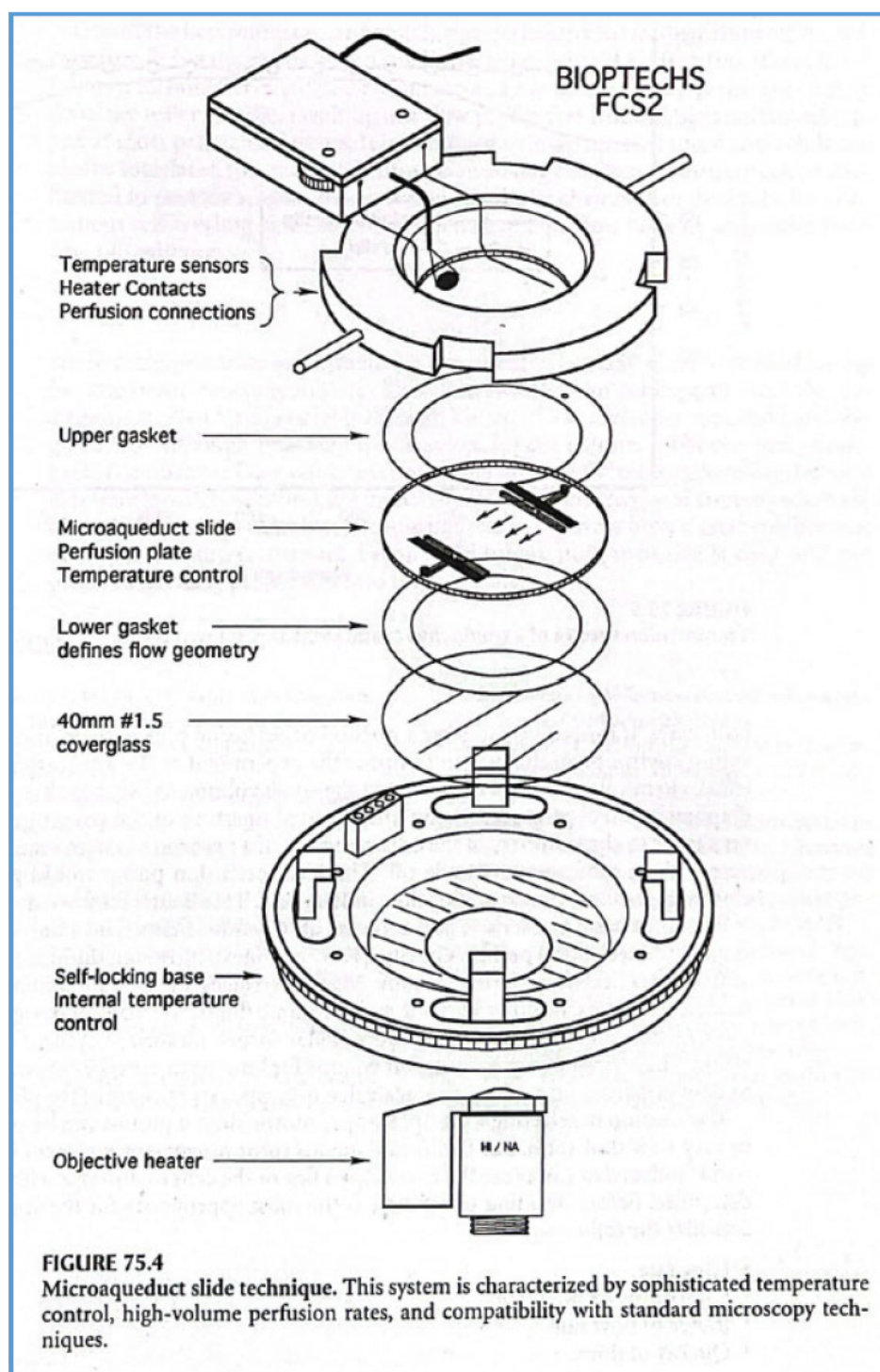
226. First, as I discuss above, Zhuang discloses a closed flow cell. *See supra* Section XI.A.3.

227. Second, Zhuang’s closed flow cell comprises a fluid inlet and outlet. As I discuss above in detail, Zhuang teaches a fluidics system designed to control the flow of the fluid “through” its exemplary flow chamber which comprises the flow cell. *See supra* Sections XI.A.1.b; XI.A.1.b.(2); *see also* EX1003.011. A POSA, from these disclosures, would have understood Zhuang to have included an inlet and outlet to get the fluid in and out of the flow cell. Indeed, a POSA would have understood the Biopetechs FCS2 chamber, as identified by Zhuang, to include an inlet and outlet port wherein the inlet port brings fluid from one side into the

chamber and the outlet port directs the fluid out of the chamber on the other side.

See, e.g., EX1006.005 (discussing “inlet and outlet ports of the chamber”).

228. An expanded view of the FCS2 flow cell system is shown below, and makes clear that the FCS2 flow cell includes the fluid inlet and outlet:



EX1015.017. This image includes a “microaqueduct slide,” and the arrows on the slide show the direction of fluid flow. The heavy black lines at the top-right of the

microaqueduct slide correspond to the inlet, while those at the bottom-left correspond to the outlet.

229. Third, Zhuang teaches that the fluid inlet is fluidically coupled to the fluid dispenser. As I discuss above, the fluid dispenser is operationally coupled to a pump configured to transfer fluid to and from the sample holder (*i.e.* flow chamber comprising the flow cell). *See supra* Section XI.A.1.b.(2). Also, as I describe above in this section, Zhuang teaches use of a fluid inlet and fluid outlet for receiving and releasing the fluid, respectively. Further as discussed above, Zhuang's fluidics system is automatic and handles processes involving fluids without user intervention, which also establishes that there are necessarily fluid inlets and outlets. *See supra* Section XI.A.1.b.(2). Thus, in my opinion, a POSA would understand from these disclosures that the fluid inlet is receiving the fluid dispensed by the fluid dispenser and is thus fluidically coupled to the said fluid dispenser.

5. Claim 43: The system of claim 36, wherein said temperature control apparatus comprises a heat sink or a Peltier element.

230. Zhuang discloses what a POSA would have understood to be the use of a heat sink, a component that absorbs heat energy. As a 1998 article on imaging of cells explains, “[i]n high N.A. imaging, it should be noted that the optical medium used to couple the objective to the coverglass will act as a heat sink.” EX1015.017 (Fig. 75.4). Zhuang discloses the use of an oil immersion objective with a

numerical aperture of 1.45. EX1003.011. A POSA would understand that this numerical aperture of 1.45 corresponds to “high N.A. imaging,” and that the oil that is used as the optical medium to couple the oil immersion objective to the coverglass is a heatsink in that it is a component that can absorb heat energy.

231. It is also my opinion that Zhuang in view of the knowledge of a POSA renders obvious the system of claim 36 where the temperature control apparatus includes a heat sink or a Peltier element.

232. As I discuss above, Zhuang teaches a temperature control apparatus. *See supra* Section XI.A.1.a.(2). Zhuang further teaches stabilizing its sample and fluid reagents flowing to sample by maintaining the temperature of apparatus, sample, and reagents. *See* EX1003.011. (“The objective was heated to 37°C with a Biopetechs objective heater.”); *see also id.* (“Cells were plated on 22-mm, #1.5 coverslips (Biopetechs, 0420-0323-2) at 350,000 cells per coverslip and incubated at 37°C...”); *id.* (“One milliliter of 10 nM of the appropriate fluorescently labeled readout probe in readout hybridization buffer...was flown across the sample, flow was stopped, and the sample was incubated for 15 minutes.”). As discussed above, a POSA would have further understood that components having thermal mass provided in the system would help maintain suitable temperatures, including by absorbing or otherwise transferring away heat energy.

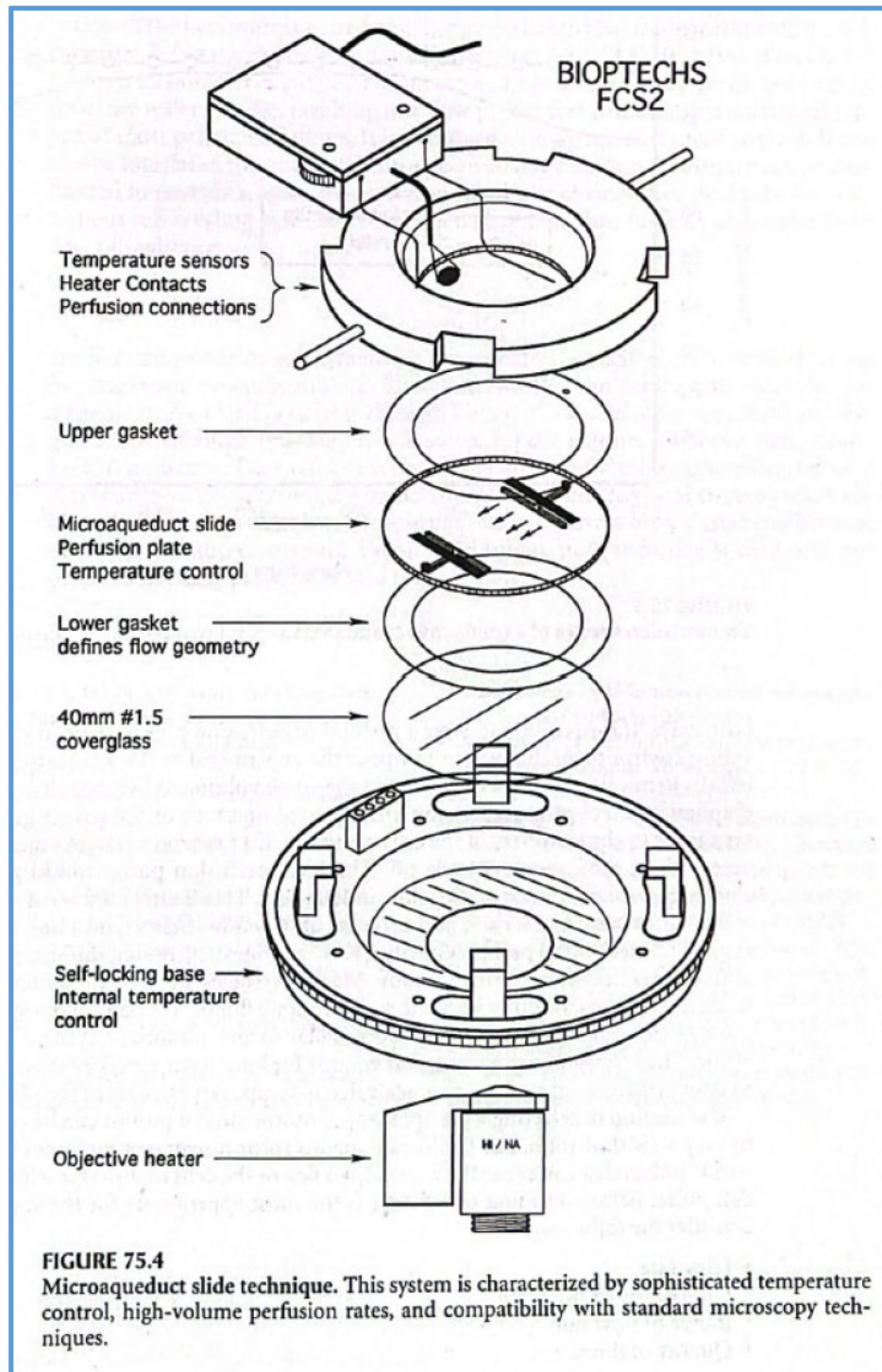
233. A POSA would also have understood that heat sinks and Peltier elements were well known and that both could be used to achieve temperature control. A POSA thus would have understood from Zhuang's disclosures relating to controlling temperature that a heat sink and/or Peltier element could be used to control temperature. As explained, a POSA would have been wholly familiar with heat sinks and Peltier elements and their use because they were commonly used before the date of the alleged invention. It is my further opinion that it would have been obvious to a POSA to include heat sinks or Peltier elements as a part of the temperature control apparatus for the benefit of improved temperature control and cooling capabilities.

6. Claim 44: The system of claim 36, further comprising a thermal control system.

234. It is my opinion that Zhuang teaches a system of claim 36 which comprises a thermal control system.

235. Zhuang discloses that the "objective was heated to 37°C with a Bioptechs objective heater." EX1003.011. In my opinion, a POSA would have known and understood that a system that controls the function of the Bioptechs objective heater being used to maintain the temperature at a particular temperature, such as at 37°C, includes a thermal control system responsible for controlling the temperature control apparatus to stabilize the temperature of the sample and flowed

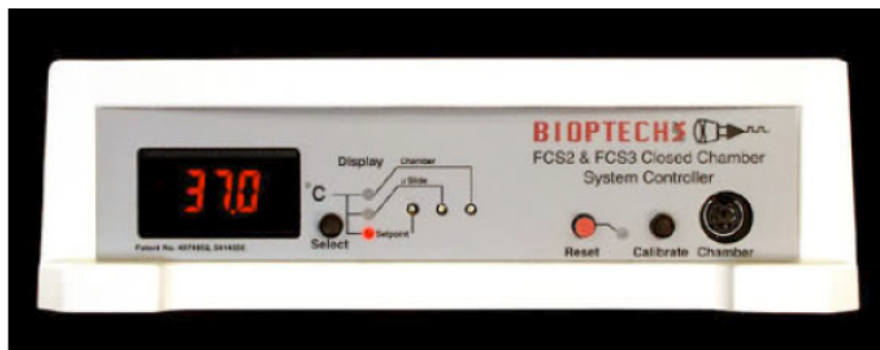
reagents. This is further evidenced by the Biopetechs FCS2 chamber system shown below:



EX1015.017 (depicting “Temperature Sensors,” “Heater Contacts,” and “Objective Heater”). A POSA would have understood the temperature sensors to detect temperature and contacts to allow for control of an objective heater, such as that depicted, in order to control the temperature, maintaining uniform temperatures.

236. A POSA would further understand a system using Zhuang’s Biopetechs FCS2 flow chamber can include a temperature controller, i.e., a thermal control system, to control the function of the temperature control apparatus, as also evidenced by the instruction manual of the FCS2 system, which depicts a controller. The instruction manual further describes the controller’s function to a POSA as including regulation of temperature, as, in relation to pressing a button to select what is displayed, it states that pressing the button to change what type of information is displayed, which can include temperature, “has no effect on the temperature regulation functions of the controller,” indicating that the controller controls temperature.

Front Panel of FCS2 & FCS3 Controller



EX1006 at Fig. 1 (depicting controller with “numeric display” displaying “37.0” while the indicator light is on for “Setpoint”), .002. The FCS2 Instructions also include an “Objective Heater Description” that describes it as “attach[ing] to the objective and plug[ging] into a separate Temperature Controller, which regulates the objective temperature within 0.1°C of SETPOINT.” *Id.* at .006.

7. **Claim 46: The system of claim 36, wherein said fluidics system further comprises one or more valves downstream of said one or more reservoirs and upstream of said fluid dispenser, wherein, upon actuation, said one or more valves facilitate dispensation of fluid to said sample holder.**

237. It is my opinion that Zhuang teaches a system in claim 36 where the fluidics system comprises one or more valves downstream of said one or more reservoirs and upstream of said fluid dispenser, wherein, upon actuation, said one or more valves facilitate dispensation of fluid to said sample holder.

238. Zhuang discloses that its fluidics system comprises “*three computer-controlled eight-way valves (Hamilton, MVP and HVX 8-5)* and a peristaltic pump (Rainin, Dyanamx RP-1.)” EX1003.011 (emphasis added).

239. As I discuss above, Zhuang also teaches that its automated fluidics system comprises a fluid dispenser that facilitates dispensation of fluid from the reservoir to the sample holder. *See supra* Section XI.A.1.b.(2). Zhuang discloses:

Sequential hybridization, imaging, and bleaching proceeded as follows. One milliliter of 10 nM of the appropriate fluorescently labeled readout probe in readout hybridization buffer...was flown across the sample, flow was stopped, and the sample was incubated for 15 minutes. Then 2 mL of readout wash buffer...was flown across the sample, flow was stopped, and the sample was incubated for 3 minutes. Two milliliters of imaging buffer...was flown across the sample. Flow was then stopped, and then approximately ~75 to 100 regions were exposed to -25 mW 642-nm and 1 mW of 561-nm light and imaged.

Id. at .011. In my opinion a POSA would have known that for Zhuang’s automated fluidics system to function, with flow of fluid from reservoir(s) to a dispenser, the system would require the use of valves between the reservoir(s) and the dispenser (apparatus to dispense fluid to a sample), and that those valves would necessarily be downstream of the reservoir(s) and upstream of the dispenser.

8. Claim 47: The system of claim 46, wherein said one or more valves comprise a rotary valve.

240. It is my opinion that Zhuang teaches a system in claim 46 where the one or more valves include a rotary valve.

241. Zhuang discloses the use of Hamilton's MVP and HVX 8-5 valves in its system. In my opinion, a POSA would have known that these valves that were in common use at the time are rotary valves because they were well known. The fact that these recited valves are rotary valves is also evidenced by Hamilton's website, which lists valves with the same designation indicating that they are rotary valves. EX1016.

242. Further, it is my opinion that a POSA would have found it obvious to use rotary valves because there are a limited number of types of valves and the use of a rotary valve specifically would have been recognized as merely the use of a known type of valve to achieve the function of a valve, switching on and off the flow, and that it would have been entirely predictable, in both the application and the result.

9. Claim 54: The system of claim 36, further comprising an objective wetting apparatus.

243. It is my opinion that Zhuang renders obvious the system of claim 36 further including an objective wetting apparatus.

244. Zhuang teaches the use of an oil immersion objective. EX1003.011.

245. Also, as discussed above, Zhuang teaches a “fully automated” system. *Id.* at .011.

246. Accordingly, a POSA would have understood Zhuang to disclose an objective wetting apparatus because it follows from the disclosed use of an oil objective that there is a mechanism for wetting the objective, i.e., for immersing the objective, when using an oil immersion objective, particularly as the system is automated. *Id.* at .011. This understanding of an objective wetting apparatus, such as for use with an oil immersion objective, is also wholly consistent with the ’554 Patent’s disclosure, including that

In aspects comprising one or more objective lenses with refractive index not matched to air, the objective lens must interface with an imaging media, such as water, oil, or other imaging buffer. In these aspects, the device may comprise a mechanism of wetting the objective lenses or otherwise creating an interface between the objective lens and the imaging medium. Certain mechanisms of lens wetting include dipping the lens into an imaging medium or otherwise dispensing an imaging medium onto the lens, such as by a syringe, needle valve, or other mechanisms known to those familiar with the art of dispensing a liquid reagent....the device dispenses a certain amount of liquid onto the objective, such as by using a syringe or needle valve. . . .

EX1001 at 22:19-36.

247. Accordingly, it is my opinion that Zhuang teaches including an objective wetting apparatus and renders obvious the system of claim 36.

10. Claim 56: The system of claim 36, wherein said two or more light sources comprise one or more lasers.

248. It is my opinion that Zhuang discloses a system of claim 36 wherein the two or more light sources comprise one or more lasers.

249. As I discuss above in Section XI.A.1.e and below in Section.XI.K.1, Zhuang teaches the use of two or more light sources. Zhuang teaches that these light sources may be lasers. *See, e.g.*, EX1003.011.

250. Accordingly, it is my opinion that Zhuang discloses a system of claim 36 wherein the two or more light sources comprise one or more lasers.

11. Claim 59: The system of claim 36, wherein said optical system further comprises one or more emission filters configured to selectively filter one or more wavelengths of light emitted from said volume to said one or more detectors.

251. It is my opinion that the Zhuang discloses the system of claim 36 wherein the optical system further comprises one or more emission filters configured to selectively filter one or more wavelengths of light emitted from the sample volume to the one or more detectors.

252. Specifically, Zhuang discloses “emission filters ET525/50m, WT5955m-2f [sic, WT595/50m-2f], ET700/75m, HQ7701p (Chroma)” in advance of the EMCCD camera. *Id.* at .011.

253. Thus, in my opinion, a POSA would understand Zhuang to disclose the optical system of claim 36 further comprising one or more emission filters configured to selectively filter one or more wavelengths of fluorescent light signals emitted from the sample volume to the one or more detectors.

12. Claim 60: The system of claim 36, wherein said optical system is mechanically, electrically, or electromechanically coupled to a controller system configured to synchronize one or more imaging events within said system.

254. It is my opinion that the Zhuang discloses the system of claim 36 wherein the optical system is mechanically, electrically, or electromechanically coupled to a controller system configured to synchronize one or more imaging events within the system.

255. Zhuang discloses that its “entire system was fully automated, so that imaging and fluid handling were performed for the entire experiment without user intervention.” EX1003.011. Zhuang further describes the use of “a multi-Gaussian-fitting algorithm” and the use of fiducial beads to align images across successive hybridization and imaging. *Id.* at .012. Zhuang also suggests the use of “super-resolution imaging and/or optical sectioning” to “allow individual molecules in these dense nucleus regions to be identified.” *Id.*

256. A POSA would have understood from these disclosures that Zhuang’s system comprises a controller configured to synchronize one or more imaging

events, that is, that there is an integrated computer controlled system to control at least the movement and function of optical components, including lenses, the movement of sample, the movement of sample-containing components, and the movement and function of detector components in a coordinated manner. Moreover, a POSA would have known that in order to automate imaging it is necessary to include a controller that is configured to synchronize imaging events, that is, to arrange or time the imaging events in the automated process. It would also have been obvious to a POSA that this controller is mechanically, electrically, or electromechanically coupled to the optical system, as it necessarily would have to be in order to synchronize the imaging events.

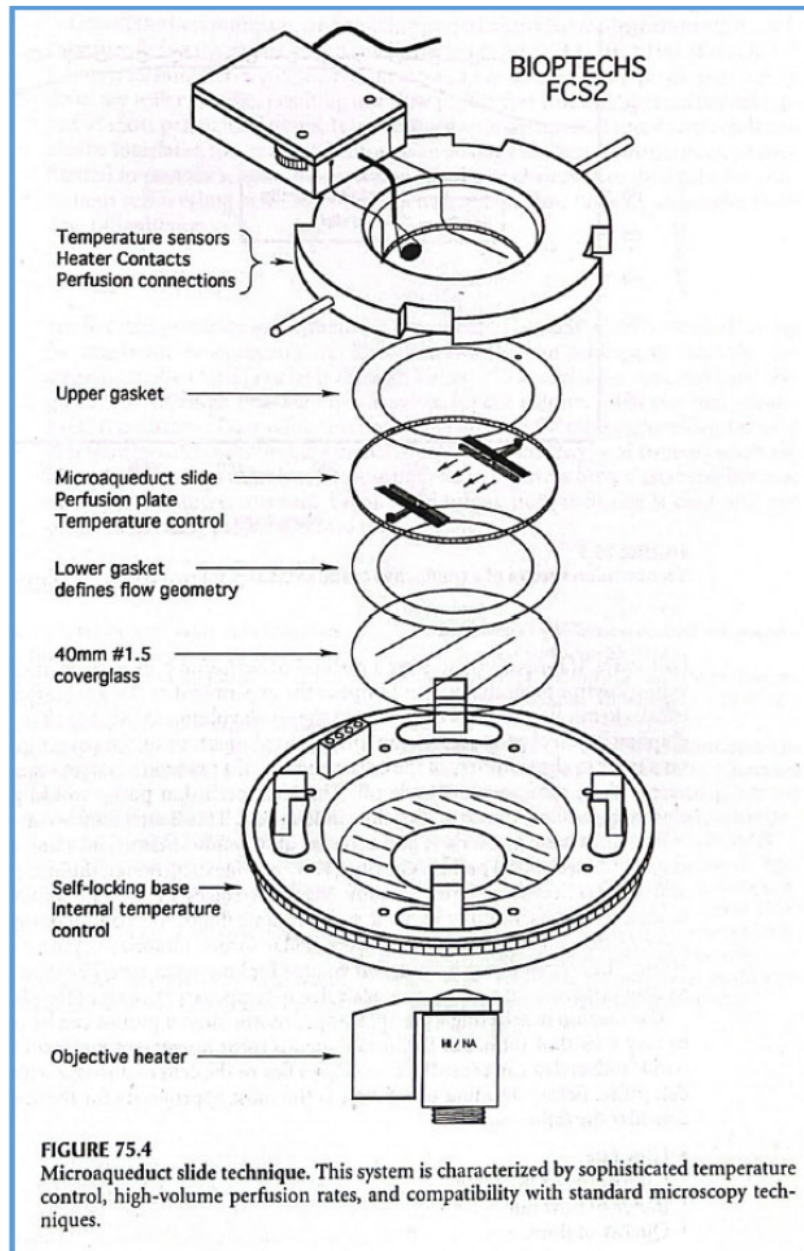
13. Claim 63: The system of claim 44, wherein said temperature control apparatus further comprises a temperature sensor configured to provide a feedback signal to said thermal control system.

257. It is my opinion that Zhuang discloses a system of claim 44 wherein the temperature control apparatus further comprises a temperature sensor configured to provide a feedback signal to the thermal control system.

258. As I describe above, Zhuang teaches a temperature control apparatus. *See supra* Section XI.A.1.a.(2). Zhuang also discloses to a POSA that an objective heater was used to maintain the temperature of the cells on the microscopic stage at 37°C. *Id.*; EX1003.011 (“The objective was heated to 37°C with a Bioptechs objective heater.”).

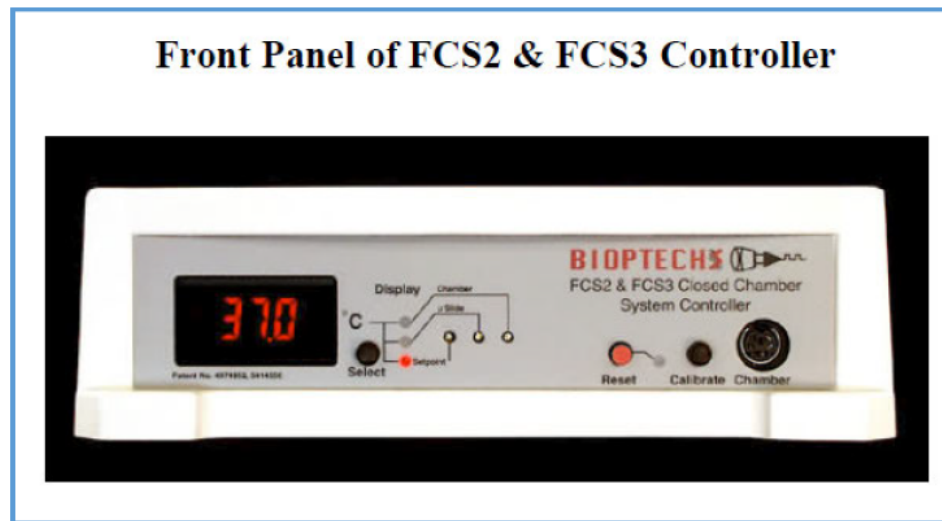
259. A POSA would have understood that the operation of the automated system described in Zhuang would have included using one or more temperature sensors to measure temperature(s) of the components (including sample and reagents present with the sample) acted upon by the temperature-control apparatus and providing that temperature information (a feedback signal) to the thermal control system, such that the system could determine whether to continue cooling or heating to regulate the temperature(s) to appropriate temperature(s).

260. Zhuang also discloses the use of a Bioprotech FCS2 flow chamber, including a temperature control apparatus. *See supra* Section XI.A.1.a.(2). Below is reproduced an expanded view of the Bioprotech FCS2 system. The top of the figure showcases conductive temperature sensors.



EX1015.017. A POSA would have understood that the temperature sensors, particularly in an automated system such as one described in Zhuang, would have been configured to provide a feedback signal to a thermal control system. This would ensure that the flow cell would be maintained at the desired temperature.

261. This understanding is confirmed by the instructions for the FCS2 system, which show that it includes a temperature controller, the front panel reproduced below.



EX1006 at Fig. 1. These instructions explain that there is a feedback loop to maintain the temperature of the flow cell and explain that it is “important that the flat side of the surface probe is making uniform contact with the microaqueduct slide because the *feedback loop that controls that microaqueduct temperature* is reliant on this thermal contact point.” *Id.* at .005 (emphasis added). Thus, in my opinion, a POSA would have found the use of a thermal sensor in Zhuang to be expressly disclosed. If the Board determines or Patent Owner argues that this is not expressly disclosed, it is my opinion that it would have been obvious to a POSA to include a thermal sensor as required.

- 14. Claim 64. The system of claim 59, wherein at least one of said one or more emission filters is configured to selectively filter light having a wavelength of about 510 nm, about 570 nm, about 620 nm, or about 680 nm to said one or more detectors from said volume to said one or more detectors.**

262. It is my opinion that Zhuang discloses a system of claim 59 where the emission filters are configured to selectively filter light having a wavelength of about 510 nm, about 570 nm, about 620 nm, or about 680 nm to detectors from the volume of the detectors.

263. As I discuss above, Zhuang teaches the use of emission filters that are configured to filter light emitted from the sample volume to the detector[s]. *See supra* Section XI.A.11. Zhuang further teaches “emission filters ET525/50m, WT59550m-2f [sic, WT595/50m-2f], ET700/75m, and HQ7701p (Chroma)” in advance of the EMCCD camera. EX1003.011. A POSA would have understood these filters to have included those that selectively filter light having wavelengths, that is, to allow passage of light having wavelengths at those recited. For example, for light having a wavelength of about 510 nm, emission filter ET525/50m would allow transmission of light over a range of 50 nm with wavelengths ranging from 500 nm to 550 nm, with a midpoint of 525 nm. Similarly, for light having a wavelength of about 680 nm, emission filter ET700/75m would allow transmission of light over a range of 75 nm with wavelengths ranging from ~663 nm to ~738 nm, with a midpoint of 700 nm.

264. Moreover, it is also my opinion that it would have been obvious to a POSA to have used emission filters that are configured to selectively filter light having a wavelength of about 510 nm, about 570 nm, about 620 nm, or about 680 nm to detectors from the sample volume.

15. Claim 65: The system of claim 36, wherein said two or more light sources excite fluorescence in unique color channels such that each light source of said two or more light sources is configured to excite fluorescence in a unique color channel of said unique color channels.

265. It is my opinion that Zhuang discloses or teaches a system of claim 36 where the two or more light sources excite fluorescence in unique color channels such that each of the light sources is configured to excite fluorescence in a unique color channel of the unique color channels.

266. As I discuss above, Zhuang discloses the use of two or more light sources. *See supra* Section XI.A.1.c.(2). Zhuang also discloses the use of unique color channels for imaging and determination of nucleic acid molecules, fluorescent beads and nuclear counterstain, as a POSA would have understood the emitted light for each label to be of a different wavelength for detection as a distinct signal. *Id.* As an initial matter, I note that the '554 Patent teaches color channels associated with use of fluorescent dyes, including specific exemplary dyes. *See* EX1001 at 12:35-37 ("After ligation, the beads are then imaged using four color channels (FITC, Cy3, Texas Red and Cy5))."

267. Zhuang discloses that in the MERFISH experiment, 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.” EX1003.011. Zhuang further discloses that the fiducial beads were “carboxylate-modified orange fluorescent beads (Life Technologies, F-8809).” *Id.* A POSA would have recognized that the emitted signals for the Cy5-labeled readout probes and the fiducial beads would differ in wavelength. As would have been understood by a POSA and confirmed by the ’554 Patent’s own teachings, these would have constituted unique color channels. As a POSA would have recognized, in Zhuang’s example, excitation of Cy5-labeled readout probes using 641 nm light would provide emission of red fluorescence light signal and excitation of the fiducial beads (Life Technologies, F-8809) with 561 nm light would provide for emission of yellow/orange fluorescence light signal, with these two different colors of light emitted constituting two different light channels. The colors of emitted fluorescence being different is evidenced by readily available resources. *See, e.g.*, EX1045 (depicting emitted spectra for the same (or essentially equivalent) labels; obtained from thermofisher.com/order/fluorescence-spectraviewer/#/ with fluorophores “Cyanine5” and “FluoSpheres orange fluorescent microspheres,” where a POSA would understand these to be the same

(or essentially equivalent)); EX1057.001 (identifying beads), .008 (depicting emission spectrum).

268. To the extent Zhuang's disclosure is argued by Patent Owner or found by the Board to be insufficient, this claim is rendered obvious by combining Zhuang with the teachings of the color channels in Cai. As described above, Cai discloses the use of a four-color FISH with yellow, green, blue, and purple probes. EX1004 at 9:45-48. Each of these would correspond to a unique color channel combination and a POSA would understand many commercial variations in dyes, light sources and filters are possible to achieve two, three or four spectrally separable color channels.

269. A POSA would have been motivated to combine the teachings of multiple unique color channels in Cai with Zhuang's teachings because they would have understood that increasing the number of color channels would provide increased flexibility as to generating a greater number of distinct signals in each hybridization and imaging cycle. Generating a greater number of distinct signals in each cycle, a POSA would also have understood, would provide the benefits of obtaining the same amount of information in less time and with better preserved sample, i.e., a sample that has been subjected to less process that damages it, than could be obtained using a single light source providing a single color of light. *See also* EX1003.008 ("Although an increase in the number of bits by adding more

hybridization rounds would increase the data collection time and potentially lead to sample degradation, these problems could be mitigated by using multiple colors to readout multiple bits in each round of hybridization.”). Accordingly, it is my further opinion that even if Zhuang had not expressly taught using two or more light sources to excite fluorescence in unique color channels, it would have been obvious to a POSA to have done so with a reasonable expectation of success.

16. Claim 66: The system of claim 65, wherein each optically-encoded signal of said plurality of optically-encoded signals comprises at least one of said unique color channels.

270. It is my opinion that Zhuang discloses to or teaches a POSA a system of claim 65 where the each optically-encoded signal of the plurality of optically-encoded signals comprises at least one of the unique color channels.

271. As I discuss above, Zhuang discloses a system comprising unique color channels. *See supra* Section XI.A.15. Zhuang also discloses a system comprising optically-encoded signals that are associated with particular nucleic acid molecules. *See supra* Sections XI.A.1.e.(2) and XI.A.1.e.(3). Further, as I describe above in Section XI.A.1.e.(1), the optically-encoded signals are generated using light signals associated with particular sequence that, cumulatively, are used to identify the presence of nucleic acid molecules of interest in the sample, as well as location of these nucleic acid molecules. In my opinion, a POSA would have

understood that the optically-encoded signals comprise at least one unique color channel.

272. Additionally, to the extent that it is argued that Zhuang does not disclose this limitation, in my opinion, it would have been obvious to a POSA to combine the relevant teachings from Cai that I describe above to arrive at optically encoded signals comprises at least one of the unique color channels. *See supra* XI.A.15. In this case, the barcodes comprise light signals with different colors, which are in turn different unique color channels.

17. Claim 67. The system of claim 36, wherein at least one of the plurality of optically-encoded signals comprises two or more colors detected in series.

273. It is my opinion that Zhuang discloses the system of claim 36 where at least one of the optically-encoded signals comprises two or more colors detected in series.

274. As I discuss above, Zhuang teaches a plurality of optically-encoded signals comprising two or more colors of light detected by one or more detectors in series. *See supra* Section XI.A.1.e.(2). It is my opinion that a POSA would have understood that Zhuang discloses or suggests that at least one of the plurality of optically-encoded signals includes two or more colors of light, detected in series. In particular, Zhuang discloses using “light having different wavelengths...and the sequence of wavelengths used to excite the sample,” in conjunction with images

obtained, which a POSA would have understood as suggesting or teaching a system in which different colored light signals would have been generated in series. *Id.* Further, a POSA would have understood that, having generated and detected two or more light signals of different color in series, the optically-encoded signals would comprise two or more colors detected in series.

18. Claim 68: The system of claim 36, wherein said two or more light sources comprise four light sources.

275. It is my opinion that the combination of Zhuang and Cai renders claim 68 obvious.

276. As discussed above, Zhuang teaches the use of multiple light sources. *See supra* Section XI.A.1.c.(2) and Section XI.A.1.e.(2).

277. Further, Cai teaches four-color seqFISH. EX1004 at 9:28-48 (*e.g.*, “[c]omposite four-color FISH Data from 3 rounds of hybridizations on multiple yeast cells.”) In my opinion, a POSA would have understood four-color seqFISH to require four light sources.

278. As I describe above, this is particularly the case when laser light sources are used, as taught by Cai’s listing of laser-excitabile dyes, because lasers emit monochromatic light. Accordingly, a POSA would have understood Cai to teach using four or more light sources and, to the extent it might be deemed not to, a POSA would have found it obvious to include four or more light sources, such as lasers, to

provide four or more colors of light for the benefit of greater diversity of signals and better spectral separation for generating distinct barcodes.

279. A POSA would have been motivated to combine the teachings of multiple colors in Cai with Zhuang's teachings because they would have understood that increasing the number of excitation light sources providing different colors of light would provide increased flexibility as to generating a greater number of distinct signals in each hybridization and imaging cycle. Generating a greater number of distinct signals in each cycle would provide, a POSA would have understood, the benefits of obtaining the same amount of information in less time and with better preserved sample, i.e., a sample that has been subjected to fewer processes that damage it, than could be obtained using a single light source providing a single color of light. *See also* EX1003.008 ("Although an increase in the number of bits by adding more hybridization rounds would increase the data collection time and potentially lead to sample degradation, these problems could be mitigated by utilizing multiple colors to readout multiple bits in each round of hybridization."). Accordingly, it is my further opinion that even if Zhuang had not expressly taught using four or more light sources to deliver two or more colors of light to the sample volume, it would have been obvious to a POSA to have done so with a reasonable expectation of success.

19. Claim 69: The system of claim 36, wherein said optical system is configured for wide-field microscopy.

280. It is my opinion that Zhuang discloses a system of claim 36 where the optical system is configured for wide-field microscopy.

281. Specifically, Zhuang teaches that its optical system is configured for wide-field microscopy, particularly for relatively large and flat cells. EX1003.012 (“Because we imaged cells using *wide-field imaging* with oblique-incidence illumination....”) (emphasis added).

B. Ground 2: Claim 40 Would Have Been Rendered Obvious By The Combination of Zhuang, Cai, And Shen In Further View of Xiao and Ehrlicher

282. It is my opinion that Zhuang, Cai, and Shen in view of Xiao and Ehrlicher renders obvious the system of claim 37 where the flow cell is an open flow cell.

283. As I discuss above, Zhuang expressly identifies using the “Biotech FCS2 flow chamber” in its system. *See supra* Section XI.A.3.

284. Zhuang, Cai, and Shen with Xiao and Ehrlicher render the system with an open flow cell obvious. As described above, Xiao discloses that the Biotech FCS2 flow chamber can be used in an open-dish format, that is, as an open flow cell where the chamber is not sealed. EX1027.099; *see supra* Section IX.D. Ehrlicher similarly discloses the use of the Biotechs FCS2 flow chamber and that it offers an

open design, as well as a closed design, and that it can be used in either mode as required. EX1028.002, .009, Fig. 2, .011-.012; *see supra* Section IX.E.

285. It is my opinion that a POSA would have been motivated to combine the teachings of Zhuang with the teachings of Xiao and Ehrlicher regarding the function and use of the Biotech FCS2 flow chamber in an open flow cell mode because these references more fully explain the operation and use of the flow chamber disclosed in Zhuang, particularly the use of an open flow chamber as it would be applied in monitoring, for example, fluid exchange and temperature control in live cells that would have been of interest to a POSA imaging fixed cells also. EX1027.098-.099; EX1028.011-.012. Further, a POSA would have been motivated to use an open flow chamber for the greater ease of access to the sample volume, including to allow use of a water dipping objective and micromanipulation at high magnification, which use would have been well-known to a POSA. *See, e.g.*, EX1014.007 (evidencing recognized benefits in 2004). Further, a POSA would have had a reasonable expectation of success in doing so because the prior art, including Xaio and Ehrlicher, teach doing so was well known in the art.

C. Ground 3: Claims 41 and 70 Would Have Been Rendered Obvious By The Combination of Zhuang, Cai, And Shen In Further View Of Tirichine

286. Claim 41 depends from independent claim 36, reciting the further limitation that the “objective is a water immersion objective”. Claim 70 depends

from claim 36 and recites “wherein a numerical aperture of said objective is about 1.0.”

287. Zhuang discloses the use of an oil immersion objective. EX1003.011. It is my opinion that a POSA would have also understood that both oil and water immersion objectives were known in the art, and that either could be utilized. A POSA would have determined it obvious to use either type of immersion objectives depending on their suitable use to optimize image quality for particular specimens, microscope configuration, and imaging task.

288. It was also well-known that water immersion objectives could be used for FISH experiments. As early as 2009, Tirichine used a water immersion objective for a “rapid protocol for fluorescent in situ hybridization (FISH) performed on 3D isolated nuclei and thin cryosectioned leaves of *Arabidopsis thaliana*.” EX1049.001; *see also id.* at .004 (“Images were taken through a 63x ***water immersion objective*** (NA 1.2, WD 0.2 mm).”) (emphasis added). Furthermore, it was also well-known that water immersion objective had numerical apertures lower than those of oil immersion objectives and that were roughly 1.0. Tirichine, for instance, discloses a numerical aperture of 1.2, which is about 1.0 and is consistent with the typical value of the numerical aperture for a water immersion lens. *Id.*

289. With it known that both oil immersion and water immersion objectives could be used, in my opinion, a POSA would have been motivated to use one type

over another as a mere design choice for the experiment. Water immersion lenses with numerical apertures of about 1.0 provided several different advantages for imaging. For example, it was well-known long before 2015 that a water immersion lens “may achieve better images from a thick, largely aqueous specimen due to the better match of refractive index between immersion medium and sample.” EX1012.006. Likewise, a POSA would have found it useful to utilize a water immersion objective while performing experiments where deeper imaging into a sample and also working with live specimen, as water or other aqueous buffer/medium is less likely to harm them. Yet another reason a POSA would also prefer water immersion objectives for their ease of use, including the ease of cleaning the apparatus after use with water as opposed to with oil, thereby making the setup and transition from one experiment to another less time consuming.

290. Given the state of the art, it is also my opinion that a POSA would have had a reasonable expectation of success. It was well-established that FISH experiments could be performed with a water immersion lens, and MERFISH is merely a type of FISH. The differences in multiplex would not have rendered it so different that a water immersion lens would not have been expected to work as it has for traditional FISH.

291. Thus, it is my opinion that use of any would have been obvious to a skilled artisan in the course of weighing the benefits of one type versus the other for a given experiment.

D. Ground 4: Claims 42 and 70 Would Have Been Rendered Obvious By The Combination of Zhuang, Cai, And Shen In Further View Of Brileya

292. Claim 42 depends from independent claim 36, further reciting that the “objective is a water dipping objective”. Claim 70 depends from claim 36 and recites “wherein a numerical aperture of said objective is about 1.0.”

293. As I discuss above, Zhuang discloses an oil immersion objective. *See supra* Section XI.A.1.c.(1). It is my opinion that a water dipping objective was also well-known to a POSA, which is named as such because the objective lens is dipped into the fluid of an open flow cell.

294. As I describe above in Section XI.C, however, other types of objectives were known to be used in FISH. Brileya, used a water dipping objective to perform confocal laser scanning microscopy in 3D-FISH to investigate biofilms. EX1039.003 (“Polyacrylamide-embedded whole biofilm for 3D-FISH and fluorescently stained hydrated biofilm were imaged on a Leica TCS SP5 II upright confocal laser scanning microscope using a 63x 0.9 NA long working distance (2.2 mm) water dipping objective (Leica Microsystems, Exton, PA, USA).”). Furthermore, it was also well-known that water dipping objectives, like water

immersion objectives, had numerical apertures lower than those of oil immersion objectives and that were roughly 1.0. Brileya, for instance, discloses a numerical aperture of 0.9, which is about 1.0 and is consistent with the typical value of the numerical aperture for a water dipping objective.

295. Additionally, as discussed above, Zhuang teaches the use of various flow cells including a closed or open flow cell. *See supra* Sections XI.A.3 and XI.B. A POSA would have been encouraged to use the open flow cell with a water dipping objective for reasons cited above.

296. In my opinion, a POSA would have been motivated to combine the relevant teachings from Brileya with Zhuang based on an experimental design choice. As of the earliest priority date of the '554 Patent, it was well known and routine in the art to use different kinds of objectives for different purposes for an experiment. A POSA interested in long term observation of a sample would have found it routine and obvious to use a water dipping objective which a POSA would have understood is advantageous to allow micromanipulation at extremely high magnification, as well as to allow ease of access of a sample to oxygen in the air, as a water dipping objective can be used with an open flow cell. *See, e.g.*, EX1014.007.

297. Further, a POSA would have had a reasonable expectation of success in combining the use of a water dipping objective as taught in Brileya with the

systems and methods for MERFISH taught in Zhuang. As Brileya demonstrates, the use of a water dipping objective with FISH was known. While Brileya may have aimed to investigate biofilms, given the goal of the study the biofilm would have been understood to be similar to a tissue sample, in that it is composed of different types of cells with different molecular markers. Thus, a POSA would have expected to be successful in applying the 3D-FISH techniques to a tissue sample as taught in Zhuang and Cai.

298. Thus, it is my opinion that the use of a water dipping objective would have been obvious to a POSA to obtain the benefits of the water dipping objective such as allowing micromanipulation at extremely high magnifications.

E. Ground 5: Claim 45 Is Unpatentable as Obvious Over Zhuang, Cai, and Shen In View Of Fang-Yen and Further in View of the Knowledge of a POSA

299. It is my opinion that the combination of Zhuang and Fang-Yen teaches and renders obvious the system of claim 36 where the pump is a syringe pump.

300. As I discuss above, Zhuang teaches the use of pumps to transfer fluid to and from the sample for sequential hybridization and imaging. *See supra* Section XI.A.1.b.(2). While Zhuang more explicitly discloses the use of a peristaltic pump, it is my opinion that the combination of Zhuang and Fang-Yen teaches the use of a syringe pump. Fang-Yen is a reference that describes a microscope utilized for a type of 3D imaging of biological samples, i.e.

Tomographic Phase Microscopy (TPM) that includes components such as a flow chamber, pumps and valves. EX1029.002.

301. Specifically, Fang-Yen discloses injecting the fluid into the sample holder “using either a manual syringe or a syringe pump (Harvard Apparatus (Holliston, Massachusetts) PHD 22/2000).” *Id.*; *see also id.* (“[u]sing the syringe pump, the hyperosmotic solution was injected into the chamber at a rate of 1.5 mL/min.”) Further, Fang-Yen discloses utilizing this syringe pump to introduce fluids into a “flow chamber [custom made or Biopetechs (Butler, Pennsylvania) FCS2].” *Id.*

302. Based on these disclosures in Zhuang and Fang-Yen, it is my opinion that it would have been obvious to a POSA to combine the teachings in the two references. As an initial matter, I consider Zhuang and Fang-Yen to be analogous art. Both references are in the field of optical microscopy and fluorescence imaging broadly, and both references teach introduction of fluid reagents into the sample for sequential imaging. EX1003.011 (“One milliliter of 10 nM of the appropriate fluorescently labeled readout probe in readout hybridization buffer...was flown across the sample, flow was stopped, and the sample was incubated for 15 min. Then 2mL of readout wash buffer...was flown across the sample, flow was stopped, and the sample was incubated for 3 min. Two milliliters of imaging buffer...was flown across the sample. Flow was then stopped, and then

~75 to 100 regions were exposed to ~25 mW 642-nm and 1mW of 561-nm light and imaged.”); EX1029.002-.003 (“The culture medium was injected into the chamber using either a manual syringe or a syringe pump (Harvard Apparatus (Holliston, Massachusetts) PHD 22/2000). A valve was used to switch the input to the flow chamber to either a culture medium containing 0.5% acetic acid (for acetic acid experiments) or a hyperosmolar phosphate-buffered saline solution (for osmolarity experiments). Using the syringe pump, the hyperosmotic solution was injected into the chamber at a rate of 1.5 mL/min. We continuously acquired tomograms while the new medium was added.”) Thus, a POSA would have been aware of both references.

303. Further, a POSA would have recognized that the combination of Zhuang with Fang-Yen would be simply substituting one known element for another to obtain predictable results. Zhuang discloses “[s]equential hybridization, imaging and bleaching” as one of the steps in its method. EX1003.011. A POSA would have recognized that syringe pumps were commonly used and would be valuable for Zhuang’s system and method because it allows for controlled and precise delivery of fluid reagents or buffers into a sample, enabling targeted and sequential hybridization, imaging and bleaching. A POSA would know that this controlled delivery is crucial for achieving high-resolution imaging as it provides a stable environment and minimal disruptions to cell physiology. A POSA would

also be well aware that Fang-Yen's "syringe pump (Harvard Apparatus (Holliston, Massachusetts) PHD 22/2000)" can be remotely controlled from a computer, as is evidenced in the Harvard Apparatus Manual. EX1031.011. A POSA would find this to be a simple substitution for Zhuang's "computer-controlled peristaltic pump," which forms part of Zhuang's fully automated system. EX1003.011. Thus, a POSA would have been motivated to combine Zhuang and Fang-Yen by using a syringe pump disclosed in Fang-Yen to achieve customizable flow rates in Zhuang's system.

304. A POSA would have had a reasonable expectation of success in combining Fang-Yen and Zhuang. Both references teach using pumps to deliver fluid reagents to the identified "Bioptechs FCS2 chamber," one being peristaltic and the other syringe. A POSA would rely on a syringe pump of Fang-Yen to ensure a constant and controlled environment, minimizing disruptions during the imaging process in Zhuang. A POSA would reasonably expect this to be successful, particularly in live cell imaging, as it would allow for accurate observations of dynamic cellular processes.

305. To the extent the combination of Zhuang and Fang-Yen does not disclose a syringe pump, a POSA interested in operating Zhuang's automated system for MERFISH imaging would have been aware of using any of the commonly used pumps. For example, a POSA would have recognized that syringe pumps were

commonly used and also know that the use of a syringe pump is preferable for a precise and controlled delivery of fluids, such as in the case of microinjections. A POSA would have recognized the use of syringe pumps as conventional and routine tools of the relevant art.

F. Ground 6: Claim 48 Is Unpatentable as Obvious Over Zhuang, Cai, And Shen In View Of Richard and Further in View of the Knowledge of a POSA

306. It is my opinion that the combination of Zhuang and Richard teaches and renders obvious the system of claim 46 wherein said one or more valves comprise a solenoid or a microfluidic valve.

307. As I discuss above, Zhuang discloses the use of valves to control the flow of fluid to and from the sample. EX1003.011. While Zhuang more explicitly discloses the use of “computer-controlled eight-way valves (Hamilton, MVP and HVXM 8-5),” it is my opinion that the combination of Zhuang and Richard teaches a solenoid valve. Richard is a reference that discusses single cell imaging of cortical neuronal cells to investigate their cellular mechanism for lipoic acid treatment. EX1030.003. To carry out such imaging, it utilizes well known elements such as an inverted microscope, flow chamber and valves for fluid transfer. *Id.*

308. Richard discloses interchanging perfusion solutions to and from the sample “using solenoid valves ((Valvelink 8 controller, Automate Scientific,

Berkeley, CA, USA).” *Id.* Richard further discloses that these solenoid valves are used to transfer the fluid to “a laminar flow chamber (FCS2; Bioprotechs, Butler, PA, USA).” *Id.*

309. It is my opinion that it would be obvious to a POSA to combine the above teachings in Zhuang and Richard. Both Zhuang and Richard are in the same field of single cell imaging and teach introduction of fluid reagents into an identical flow chamber i.e., “Bioprotechs FCS2” for imaging of the sample, with the help of valves. EX1003.011 (“The sample coverslip was assembled into a Bioprotech’s FCS2 flow chamber, and the flow through this chamber was controlled via a homebuilt fluidics system composed of three computer-controlled eight-way valves (Hamilton, MVP and HVXM 8-5) and a computer-controlled peristaltic pump (Rainin, Dynamax RP-1).”); EX1030.003 (“Individual coverslips were then placed in a laminar flow chamber (FCS2; Bioprotechs, Butler, PA, USA) mounted on an inverted epifluorescence microscope (Axiovert 200; Zeiss) equipped with a CCD digital camera (Axiocam MRm; Zeiss). Perfusion solutions were gravity fed at 2ml/min and were interchanged using solenoid valves (Valvelink 8 controller, Automate Scientific, Berkeley, CA, USA).”). Thus, a POSA would have been aware of both references.

310. It is also my opinion that a POSA would have recognized that the combination of Zhuang with Richard would be simply substituting one known

element for another to obtain predictable results. Zhuang discloses “[s]equential hybridization, imaging and bleaching” as one of the steps in its method. EX1003.011. A POSA would have recognized that solenoid valves were commonly used and would have been valuable for Zhuang’s system and method because they allow for accurate and rapid control over the flow of fluid reagents or buffers into a sample, enabling precise delivery and consistent sequential hybridization, imaging and bleaching. A POSA would have known that this controlled delivery is crucial for achieving high-resolution imaging as it provides a stable environment and minimal disruptions to cell physiology. Further, a POSA would also have been well aware that Richard’s “solenoid valves (Valvelink 8 controller, Automate Scientific, Berkeley, CA, USA)” can be remotely operated via a computer. A POSA would find this to be a simple substitution for Zhuang’s “computer-controlled eight-way valves (Hamilton, MVP and HVXM 8-5),” which forms part of Zhuang’s fully automated system. EX1003.011. Thus, a POSA would have been motivated to combine Zhuang and Richard by using solenoid valves disclosed in Richard to achieve precise delivery of fluids in Zhuang’s system.

311. A POSA would have had a reasonable expectation of success in combining Richard and Zhuang. Both references teach using valves to deliver fluid reagents to the identified “Bioptechs FCS2 chamber.” A POSA would have relied on the solenoid valves of Richard to maintain experimental consistency, minimizing

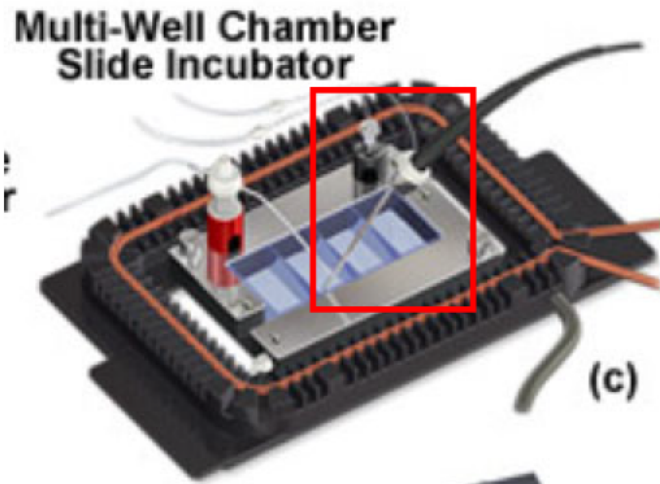
any disruptions during the imaging process in Zhuang. A POSA would reasonably have expected this to be successful, particularly in live cell imaging, as it would allow for accurate observations of dynamic cellular processes.

312. To the extent the combination of Zhuang and Richard does not teach a solenoid valve, again, a POSA interested in operating Zhuang's automated system for MERFISH imaging would have been aware of using any of the commonly used valves and would have also understood that these valves may comprise a solenoid or microfluidic valve. As of the priority date of the '554 Patent, a POSA would have known that these valves were well-known and readily accessible. Further, a POSA would have found it obvious to use either a solenoid or microfluidic valve depending on the specific requirements of the experiment. For example, a POSA would have found it useful to utilize a solenoid valve for its versatility and rapid control of broad range of fluids while maintaining experimental consistency compared to the use of microfluidic valve which is useful for applications demanding minimal fluid flow and the ability to manipulate smaller quantities with higher precision. A POSA would have recognized the use of solenoid and microfluidic valves as conventional and routine tools of the relevant art, and have had a reasonable expectation of success using them.

G. Ground 7: Claim 49 Would Have Been Rendered Obvious By The Combination of Zhuang, Cai, And Shen In Further View of Nikon

313. Claim 49 depends from claim 40 and recites that “said fluidics system further comprises an aspiration system comprising [] an aspiration tube configured to remove fluid from said open flow cell and a pressure source operatively coupled to said aspiration tube.” It is my opinion that Zhuang in view of Nikon renders this claim obvious.

314. An aspiration system configured to remove fluid from an open flow cell was a well-known approach for reagent exchange with numerous benefits, including rapid and controlled removal of fluids without disturbing the biological sample. For instance, Nikon, an educational webpage published in and before 2015 by Nikon and entitled “Culture Chambers for Live-Cell Imaging,” describes the use of an aspiration system in connection with live-cell imaging: “Among the many unique features of this incubator system are a magnetic base for ease in microscope configuration and a specially designed aspirator to remove perfusate with minimal disturbance of fluid level.” EX1043.001.007.



Id. at .006 (Fig. 6(c) (annotated)). A POSA would have understood that the aspirator comprises an aspiration tube configured to remove fluid from the flow cell (red box) and a pressure source operatively coupled to the aspiration tube—particularly, a vacuum source that withdraws the perfusate from the flow cell.

315. A POSA would have been motivated to use an aspiration system in 2015 because the system was known to work with open flow cells to provide rapid fluid removal without disturbing the biological sample as reflected in Nikon. A POSA likewise would have had a reasonable expectation of being able to successfully use an aspiration system in this manner in 2015 because the system had been successfully used to remove fluid as shown in Nikon.

H. Ground 8: Claims 50 And 51 Would Have Been Rendered Obvious By The Combination of Zhuang, Cai, And Shen In Further View of Nikon and Skafte-Pedersen.

316. Claim 50 depends from claim 49 and recites that “said aspiration system further comprises a motor operatively coupled to said aspiration tube, wherein said

motor is configured to adjust a motion axis of said aspiration tube to control an interface between said aspiration tube and said open flow cell.” Claim 51 depends from claim 50 and recites the use of a stepper motor. It is my opinion that Zhuang in view of Nikon and Skafte-Pedersen renders these claims obvious.

317. As discussed above, it would have been obvious to a POSA to use an aspiration tube configured to remove fluid from the open flow cell. *See supra* XI.G. To achieve this function, a POSA would have been motivated to adjust the position of the aspiration tube relative to the open flow cell (*i.e.*, motion axis) to control an interface between the aspiration tube and the open flow cell in order to control the amount of fluid to be aspirated. For instance, if the entirety of the fluid is to be aspirated, the interface should be set to the bottom of the flow cell; if half of the fluid is to be aspirated, the interface should be set to the half depth of the flow cell. A POSA would have understood that the use of a motor—an extremely common component in fluidic systems—would have allowed this process to be automated, which furthers the teaching of an automated system in Zhuang. *See* EX1003.011 (“The entire system was fully automated, so that imaging and fluid handling were performed for the entire experiment without user intervention.”). A POSA would have had a reasonable expectation of success in this approach as using a motor to adjust the position of a fluidic component was a common and well-understood approach before 2015.

318. Further, it would have been obvious to a POSA to use a stepper motor to adjust the position (*i.e.*, motion axis) of the aspiration tube. A stepper motor was a well-known component for fluidic systems in 2015, and offers numerous benefits, such as being easy to build and providing precise and smooth control. For instance, an article introducing a “self-contained, programmable microfluidic cell culture system with real-time microscopy access” in 2012 describes the use of stepper motor:

To achieve a smooth motion at such low speeds combined with a minimum footprint and weight for microscopy compatibility a geared stepper motor is used. The chosen model, which has a cross sectional area smaller than the remaining pump parts, is a 24 step miniature stepper motor (PRECISTEP AM 1524, Faulhaber, Germany) equipped with a planetary precision gearhead of reduction ratio 152:1 (15A, Faulhaber, Germany).

EX1048.004. Similar to controlling the motion of a miniature pump component, a POSA would have understood that a stepper motor was suitable for controlling the motion of an aspiration tube.

319. A POSA would have been motivated to use a stepper motor in 2015 because stepper motors were known to provide smooth positioning and be compatible with microscopy as reflected in Skafte-Pedersen. A POSA likewise would have had a reasonable expectation of being able to successfully use a stepper

motor in 2015 because these motors had long been used to control the motion of a fluidic component as shown in Skafte-Pedersen.

I. Ground 9: Claim 52 Would Have Been Rendered Obvious By The Combination of Zhuang, Cai, And Shen In Further View of Sands

320. Claim 52 depends from claim 36 and recites the use of a “Z-axis linear motor configured for Z-motion of said objective focal plane through said volume.” As I explain above in connection with claim 36, Zhuang discloses the use of a Z-axis motion control system in connection with its used of an Olympus IX-71 microscope. To the extent Zhuang does not disclose the use of a linear motor for the Z-axis control, this would have been obvious to a POSA.

321. For instance, in 2009, the brochure for Olympus microscopes described the use of microscopes that were “fully motorized” and that included a “high-precision z-motor.” EX1033.010. As of 2015, it was extremely common to use precision Z-motors in connection with microscopy.

322. A POSA would have known that the Olympus IX-71 microscope could have included a linear motor for the express purpose of imaging and different focal points. One method of autofocus taught by Shen using the Olympus IX-71, for example, is through an “electric stepper motor attachment *with linear-encoded feedback*.” EX1032.004. A POSA would have understood this to refer to a linear motor, as linear-encoded feedback would have been used to adjust a linear motor.

Thus, a POSA would have found it obvious to use a linear motor for Z-axis control in Zhuang.

323. To the extent that the use of the Olympus IX-71 outfitted for linear motor control is considered insufficient, however, the inclusion of such a motor would have been obvious. For instance, Sands described a system for automated imaging of extended tissues using confocal microscopy to generate three dimensional images of tissue. *See* EX1046.001, .005 (Fig. 3). To move the focal plane through the sample, the system utilized a linear motor to effectuate motion in the Z-direction:

The three-axis translation stage was constructed using two Aerotech ALS5000-based linear stages giving 1,000- and 200-mm travel in the X and Y axes, respectively, and a custom-built Aerotech vertical lift stage providing 76-mm travel in the Z axis. Each stage was driven using a noncontact, direct drive linear motor, with an addressable step resolution of 0.1 μm .

Id. at .002.

324. A POSA would have been motivated to use a linear motor in 2015, as in Sands, because linear motors were known to provide a wide range of motion with precise positioning that would enable three dimensional-imaging of biological samples, where Shen teaches a “motorized x-y stage with linear encoding feedback accuracy” is necessary to “carry out digital autofocus in a microscopy scan.”

EX1032.002.004. A POSA likewise would have had a reasonable expectation of being able to successfully use a linear motor for this purpose in 2015 because such linear motors had long since been used in this manner, as shown in Sands.

J. Ground 10: Claim 53 Would Have Been Rendered Obvious By The Combination of Zhuang, Cai, And Shen In Further View of Sands And Brooker

325. Claim 53 depends from claim 52 and further recites the use of a “linear encoder configured to provide Z-motion feedback to said Z-axis linear motor.” Briefly, a linear encoder encodes position, and the encoded position can be provided to the Z-motor of claim 52 so as to adjust the position.

326. To the extent Zhuang, Cai, and Shen in view of Sands does not include a linear encoder with Z-motion feedback, this would have been obvious further in view of Brooker. As I explain above, Zhuang teaches autofocusing, explaining that “[c]onstant focus was maintained throughout the imaging process with a home-built, autofocusing system.” EX1003.011. To maintain focus with a microscope, a person of skill in the art would have been motivated to utilize a linear encode position and provide position information to the motor for the purpose of adjusting position. This was a standard manner of providing autofocusing, as reflected, for instance, in Brooker, which teaches an autofocusing apparatus for a microscope. EX1040. To implement the autofocusing mechanism, Brooker explains that “position

feedback is facilitated using, in one embodiment, linear encoder scales embedded within the stage construction.” *Id.* at ¶ 23.

327. Yet another example, as I describe above, is taught in Shen. To autofocus, Shen suggested configuring the Olympus IX-71 microscope with an “electric stepper motor attachment *with linear-encoded feedback.*” EX1032.004 (emphasis added). A POSA would have known that to successfully autofocus a microscope, they could use a linear-encoded feedback system to precisely control the distance between the objective and the sample. Thus, a POSA would have found it obvious to use this system.

328. A POSA seeking to implement an autofocusing system of the type set forth in Zhuang in 2015 would have been motivated to use a linear encoder to provide feedback to a Z-motor because such systems were standard components that were known to be capable of effectively providing feedback in the context of microscope autofocusing, as reflected in Brooker. And, a POSA would have expected success in using such an approach for precisely the same reason.

K. Ground 11: Claims 55, 57, And 58 Would Have Been Rendered Obvious By The Combination of Zhuang, Cai, And Shen In Further View Of Wessels

1. Claim 55

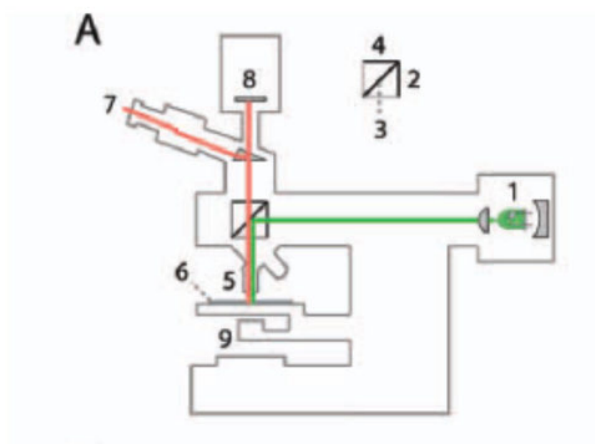
329. Claim 55 depends from the independent claim 36 and further recites “wherein said two or more light sources comprise one or more light emitting diodes (LEDs).”

330. To the extent that Zhuang, Cai, and Shen are found not to disclose the use of LEDs as a light source, it is my opinion that the inclusion of LED lights would have been obvious in further view of Wessels. As I describe above, Zhuang and Cai both teach the use of multiple light sources. *See supra* Section XI.A.1.(c).(2); EX1003.011.

331. The use of LEDs as a light source to excite fluorescent moieties for fluorescence microscopy was well-known long before 2015. Wessels is a review article from 2012 that discloses that LEDs were “ideal light sources for fluorescence microscopy” due to their high durability and because they do not need expensive electronics, they can be switched in nanoseconds, and “they are available throughout the UV/Vis/NIR-spectrum with a narrow bandwidth.” EX1050.001 (Abstract).

332. Wessels discloses “standard fluorescence microscope[s]...for fluorescence excitation” that include an emission filter in “a conventional filter block

containing a dichroic mirror (excitation filter/dichroic mirror/emission filter)” in discussing the use of LEDs to replace an arc lamp. *Id.* at .005-.006.



Id. at Fig. 1(A) (depicting system “where a LED replaces the arc lamp at the same position. (1 LED, 2 excitation filter, 3 dichroic mirror, 4 emission filter, 5 objective, 6 specimen, 7 ocular, 8 camera, 9 green path indicates excitation light, red path indicates fluorescence light).”). Wessels also discloses high-power LEDs for fluorescence and light microscopy applications in Table 3. *Id.* at .009; *see also id.* at .003 (Table 1, listing particular substitutions of LEDs in particular devices for “fluorescence microscopy, illuminators, adapters, and integrated solutions”). Wessels describes particular advantages of using LEDs, including high durability, simplicity in not needing expensive electronics, and ability to be switched in nanoseconds. *Id.* at .001 (Abstract), .002.

333. Therefore, it is my opinion that a person of ordinary skill in the art would have understood that Wessels discloses the use of LED lights as a light source for fluorescence microscopy.

334. Further, it is my opinion that it would have been obvious to combine these teachings of the use of an LED as a light source for fluorescence microscopy with the techniques of MERFISH or seqFISH as taught in Zhuang and Cai.

335. A POSA would have been motivated to combine the teachings because, as I describe above, the advantages to LEDs over traditional technologies were well known. A POSA also would have had a reasonable expectation of success, given the understanding of the state of the art that included references teaching the use of high power LED excitation light sources for fluorescence microscopy, including for single-molecule detection and imaging. For example, a review article in 2014, “Analytical devices based on light-emitting diodes – a review of the state-of-the-art” by Bui and Hauser, teaches that even though “in fluorescence the sensitivity is directly proportional to the intensity of the excitation source,” such as exploited using lasers, “[i]t has also been shown...by different authors that single molecule detection can also be achieved with high intensity LEDs.” EX1026.010 (citing EX1054, EX1055, and EX1056). Gerhardt et al., titled “Detection of Single Molecules Illuminated by a Light-Emitting Diode,” was published in 2011 and teaches that its methods “might be extended to dyes which are presently more

relevant in biological imaging, such as Cy3, **Cy5** and other Alexa-Dyes in the red region of the spectrum” (emphasis added), where Cy5 is the dye used in Zhuong, discussed above. EX1054.004-.005. Hattori et al., titled “Single-molecule Imaging with an Inexpensive UV-LED Light Source” was published in 2009 and teaches “the use of a low-cost high-power UV light emitting diode (LED)...as a light source for single-molecule detection” and that it “provides an affordable alternative to UV laser systems” that “might open new areas of...biological systems for study.” EX1055.001. Kuo et al., “High-power blue/UV light-emitting diodes as excitation sources for sensitive detection” was published in 2004 and describes the use of “high-power blue and UV LEDs for sensitive fluorescence detection, including...single-molecule imaging.” EX1056.001 (Abstract). Bui further discloses that systems using LEDs for fluorescent excitation in microscopy, and other analytical instruments, were commercially available. EX1026.010.

336. Moreover, to the extent there might have been difficulties in using an LED light source for single-molecule imaging grounded on sensitivity where there is a single fluorophore per nucleic acid of interest, a POSA would also have recognized that using multiple fluorescently labeled probes for each target nucleic acid, as in Cai, would increase the fluorescent signal, and a POSA would, thus, have found it obvious to modify the combination to include multiple fluorescent probes to obtain increased sensitivity with a reasonable expectation of success.

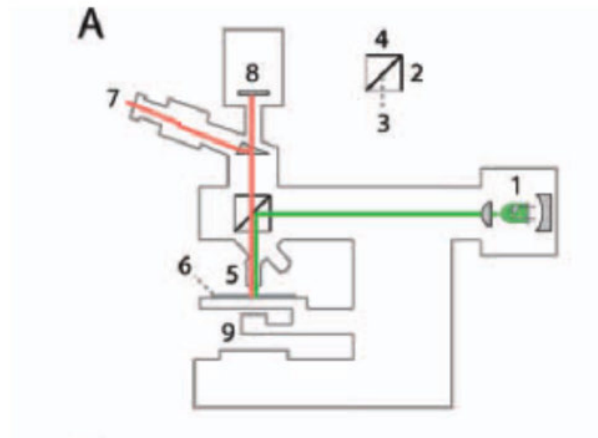
2. Claim 57

337. Claim 57 depends from the independent claim 36 and further recites “wherein said optical system further comprises one or more excitation filters configured to selectively filter one or more wavelengths of light from said one or more light sources to said volume.”

338. To the extent that Zhuang, Cai, and Shen are found not to disclose the use of excitation filters to selectively filter one or more wavelengths of light from the light sources to the volume, it is my opinion that including such filters would have been obvious in further view of Wessels.

339. As I discuss above, Zhuang discloses an optical system. *See supra* Section XI.A.1.c. Zhuang further discloses an optical system that includes apparatus to obtain substantially monochromatic light or to otherwise modify the light coming from the light source. *See* EX1003.011 (“Illumination was provided at 641, 561, and 405 nm by using solid-state lasers . . . for excitation of our Cy5-labeled readout probes, the fiducial beads, and nuclear counterstains, respectively.”).

340. Wessels discloses “standard fluorescence microscope[s]...for fluorescence excitation” that include an emission filter in “a conventional filter block containing a dichroic mirror (excitation filter/dichroic mirror/emission filter)” in discussing the use of LEDs to replace an arc lamp. EX1050.005-.006.



Id. at Fig. 1(A) (depicting system “where a LED replaces the arc lamp at the same position. (1 LED, 2 excitation filter, 3 dichroic mirror, 4 emission filter, 5 objective, 6 specimen, 7 ocular, 8 camera, 9 green path indicates excitation light, red path indicates fluorescence light).”). As discussed above, Wessels discloses the use of high-power LEDs for fluorescence and light microscopy applications in Tables 1 and 3, including advantages of LEDs. Section XI.K.1; EX1050.001, .003, .009.

341. As I describe in Section XI.K.1, a POSA would have been motivated to use LEDs because the advantages to the use of LED light systems over pre-existing technologies was well known in the art. These advantages include both broad spectrum and specific wavelength illumination, no inadvertent UV byproduct, and rapidly reaching full intensity and extinguishing. Further, as I describe above, the use of LEDs for fluorescent excitation was well-established and there were commercially available systems, such that a POSA would have had a reasonable expectation of success. *See supra* Section XI.K.1; EX1026.010.

342. In teaching the substitution of LEDs for other light sources in fluorescence microscopy applications, in systems laid out in a conventional manner, Wessels teaches the use of an excitation filter generally in fluorescence microscopy, as well as more specifically for those systems that use LEDs for light sources. It also evidences that using excitation filters in fluorescence microscopy systems was well-known and wholly conventional to provide light to the sample to be imaged for improved imaging, and that a POSA would have had a reasonable expectation of success.

343. Accordingly, in my opinion, it would have been obvious to include in the system taught by Zhuang, Cai, and Shen an optical system comprising one or more excitation filters configured to selectively filter one or more wavelengths of light from the one or more light sources to the sample volume an excitation filter as explicitly taught by Wessels.

3. Claim 58

344. It is my opinion that the combination including Wessels discloses or suggests the system of claim 57 where at least one excitation filter of the one or more excitation filters is configured to selectively filter light having a wavelength of about 480 nm, about 530 nm, about 590 nm, or about 640 nm from the one or more light sources to the sample volume.

345. As discussed above, Zhuang teaches use of one or more light sources, to provide light at 641 nm for use in exciting the Cy5-labeled readout probes. *See supra* Section XI.A.1.c.(2) and Section XI.K.2. Wessels likewise discloses excitation of fluorophores in fluorescence microscopy, including particular excitation light sources. EX1050.001 (Abstract), .006-.007. The '554 Patent discloses exemplary wavelengths preferred for excitation in the '554 Patent, and recites those in claim 58. EX1001 at claim 57, 10:8-10 (“According to another exemplary aspect, the colors of excitation light are distributed around about 480 nm, 530 nm, 590 nm, and/or 640 nm.”), claim 58 (reciting “to filter light having a wavelength of about 480 nm, about 530 nm, about 590 nm, or about 640 nm”).

346. Wessels teaches providing filters that likewise filter (i.e., allow transmission light of those same wavelengths while blocking others) for excitation in its disclosure of: (1) using excitation filters (EX1050 at Fig. 1); and (2) of light sources providing light at 470 nm, 525 nm 530 nm, 590 nm, and 650 nm (*id.* at .009 (Table 3) (listing high-power LEDs for fluorescence and light microscopy application). Specifically, Wessels discloses light sources of 530 nm and 590 nm, that are exactly the wavelengths recited in claim 58, and light sources of 470 nm, 525 nm, and 650 nm, that are “about” the remaining values of, as recited in the claim, “about 480 nm, about 530 nm, and about 640 nm, respectively. *Id.* at .009 (Table

3). As discussed above, Zhuang likewise specifically discloses an excitation light source of 641 nm, which is “about 640 nm.”

347. Accordingly, in my opinion, it would have been obvious for the one or more excitation filters in the combination to be configured to selectively filter light having a wavelength of about 480 nm, about 530 nm, about 590 nm, or about 640 nm from the one or more light sources to the sample volume.

L. Ground 12: Claim 61 Would Have Been Rendered Obvious By The Combination of Zhuang, Cai, And Shen In Further View Of The Skill And Knowledge Of A POSA

348. Claim 61 depends from independent Claim 36, and further recites that the system comprises a vibration control rigid foundation.

349. Zhuang teaches a fully automated system for imaging or determination of nucleic acids that includes a microscope stage. *See* EX1003.011. Zhuang further teaches automated steps and devices to ensure proper imaging. Zhuang also teaches that this fully automated system is part of a homebuilt system constructed around the Olympus IX-71 microscope.

350. A POSA would have understood that fluorescence microscopy systems, such as that disclosed in Zhuang and Cai, would necessarily have included a vibration control rigid foundation because effectively eliminating vibrations is necessary for obtaining suitable quality images and data. Specifically, it is essential to stabilize the system components and to isolate the microscope system

from vibrations introduced from the environment, including vibrations conducted through the building structure in which the apparatus is located. Such vibration control rigid foundations and their use is well known in the field, as well as in many others where precision measuring and testing equipment is used. The '554 Patent itself evidences this in its disclosure to include a “vibration control rigid foundation” that it depicts in Figure 3 in a manner that only informs a POSA that it is present as a foundation for other components, without providing any additional definition or detail. EX1001 at Fig. 3, 26:21, 26:43-44.

351. In my opinion, thus, a POSA would have known long before 2015 that the Olympus IX-71 microscope used in Zhuang would have included a vibration control rigid foundation as an essential component for the work conducted. This understanding is supported by evidence that the Olympus IX-71 microscope did include such a foundation, for example, looking to a 2009 product brochure for the microscope, the platform is designed so that “once defined, target positions will be perfectly kept despite vibrations and environmental changes.” EX1033.009. Further, highlighting the recognized need to eliminate vibrations, the brochure also describes a “vibration-free shutter to be mounted in the reflected or transmitted light path” and an “illumination pillar tilt mechanism with ... [a] vibration-reducing mechanism.” *Id.* at .010, .018. The Olympus IX-71 product brochure also

discloses that the platform is includes a “slim and rigid frame” to combine “optimal alignment and stability with ergonomic handling.” *Id.* at .004, .009.

352. Accordingly, in my opinion, a POSA would have known that Zhuang’s system included a vibration control rigid foundation. Therefore, Zhuang renders this claim limitation obvious.

353. To the extent, however, the Board finds or Patent Owner argues that this is not sufficient disclosure, it is my also opinion that it would have been obvious to a POSA to provide a vibration control rigid foundation that would mitigate and limit vibration of the apparatus of the system for the reasons set forth above. In other words, it would have been obvious to include a vibration control foundation in the system in order to improve image quality and that a POSA would have had a reasonable expectation of success in doing so, as it was well-known to do so for that same benefit.

M. Ground 13: Claim 62 Would Have Been Rendered Obvious By The Combination of Zhuang, Cai, And Shen In Further View Of Niman and/or Winer.

354. Claim 62 depends from independent claim 36 and further recites “a fluidics controller operatively coupled to said fluid dispenser and configured to automate or control dispensation of said one or more fluid reagents to said sample holder.”

355. As I describe above, it is my opinion that the combination of Zhuang, Cai, and Shen render Claim 36 obvious. *See supra* Section XI.A.1. It is also my opinion that the disclosures in Zhuang, particularly of a computer-controlled fluidics systems, render the additional limitation of claim 62 obvious.

356. To the extent that the Board finds or Patent Owner argues that these disclosures are insufficient to render claim 62 obvious, it is also my opinion that claim 62 would have been rendered obvious in further view of Niman and/or Winer. Niman teaches the use of the Fluigent Fluidics system, a commercially available system fluidics control system. *See, e.g.*, EX1044.003 (“Pressure-driven flow and control of differential pressures to inlets and waste outlets was achieved using a Fluigent microfluidic control system); *see also* EX1051.003 (“The inlet tubing was fit to a flow pressure control system (Fluigent MFCS-8C, Villejuif, France).”)

357. Fluidics control systems such as the Fluigent controller were common, commercially available systems. Further, these systems were commonly used in studies with fluorescence microscopy to study a single cell or in three dimensional point tracking. Niman, for example, used “total internal reflection fluorescence microscopy to characterize the time-dependent change in concentration during switching of solutions” in the single-molecule and single-cell context. EX1044.001 (Abstract). Winer, meanwhile, used the Fluigent system to present a method of “three-dimensional (3D) particle tracking” and described a defocusing

technique that provides “a simple and precise tool for determining the 3D locations of cell-sized particles in microscale flows.” EX1051.001.

358. It is my opinion that, given the commercial availability of such systems, it would have been obvious to a POSA to combine the use of such off-the-shelf systems with the methods taught in Zhuang and Cai. Particularly, a POSA would have been aware of the need for some kind of fluidics control system in automating systems of cell imaging. A POSA would have been motivated to look to commercially available systems, and would have had a reasonable expectation of success in using them given the known successes of applying such systems in single cell settings.

XII. DECLARATION IN LIEU OF OATH

I 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 1034 of Title 18 of the United States Code.

Dated: 1/24/24

By: 