

**Development of Multiplexed Imaging Strategies for
Fluorescence and Electron Microscopy**

Hwanhee Kim

A dissertation

submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2026

Reading Committee:

Joshua C. Vaughan, Chair

Daniel T. Chiu

Dan Fu

Program Authorized to Offer Degree:

Chemistry

©Copyright 2026

Hwanhee Kim

University of Washington

Abstract

Development of Multiplexed Imaging Strategies for
Fluorescence and Electron Microscopy

Hwanhee Kim

Chair of the Supervisory Committee:

Joshua Vaughan

Chemistry

Microscopy in biological research often relies on complementary analysis across multiple imaging modalities to reveal complex molecular contents and their spatial organization. Selecting the most appropriate method for a given experiment requires balancing and assessing the need for imaging speed, molecular specificity, accessibility, and resolution. In general, optical microscopy offers strong molecular specificity and multiplexed target detection, whereas electron microscopy provides superior spatial resolution. This dissertation explores two challenges in improving multiplexed imaging strategies across fluorescence microscopy and electron microscopy. Chapter 1 introduces the general principles of microscopy, conventional strategies for multiplexed imaging, and the limitations of these approaches. Chapter 2 addresses the fundamental challenge of improving single-shot spectral multiplexing and presents a polymer dot (Pdot)-based strategy for

achieving 11-color imaging through a single round of labeling, excitation, and acquisition. Chapter 3 examines current limitations in implementing labeling strategies within cryogenic electron microscopy (cryo-EM) workflows and introduces a live-cell delivery approach using the mild detergent saponin to deliver exogenous probes prior to cryofixation while potentially retaining ultrastructure. Together, this dissertation discusses new approaches for supporting biological research through powerful and accessible multiplexed imaging strategies.

Table of Contents

Introduction	10
1.1 Overview of microscopy techniques	10
1.1.1 Important applications in research and clinical settings	10
1.1.2 Comparisons between various microscopy techniques	11
1.1.2.1 Light microscopy	12
1.1.2.2 Electron Microscopy	13
1.1.3 Importance of labeling for microscopy	14
1.1.3.1 Absorption-based and fluorescence labeling strategies	15
1.1.4 Multiplexing strategies	18
1.1.4.1 Multicolor probe selection / Physical separation of wavelengths of light ...	19
1.1.4.2 Labeling workflow	23
1.1.4.3 Finding the balance between highly multiplexed images and fast turnaround time	25
1.2 Highly multiplexed spectral line-scan microscopy at single-excitation wavelength	26
1.2.1 Limitations of widely conventional sequential labeling and imaging technique	26
1.2.2 Leveraging emergence of polymer dots	28
1.3 Live-cell labeling for cryogenic electron microscopy	29
1.3.1 Limitations of heavy-metal labeling technique	29
1.3.2 Motivation for live-cell delivery of exogenous probes	30
1.4 Summary	31
1.5 References	32
11-Channel Fluorescence Microscopy Imaging with a Single Excitation Wavelength Using Polymer Dots with Spectral Line-scan Microscopy	39
2.1 Abstract	39
2.2 Introduction	40
2.3 Methods	43
2.3.1 System design	43

2.3.2 Detection parameter and calibration.....	44
2.3.3 Spectral unmixing	45
2.3.4 Sample preparation.....	48
2.4 Results	49
2.5 Conclusions and discussion	55
2.6 References	57
Live-cell Delivery of Nanogold-conjugated Exogenous Probes for Cryo-EM Labeling and Detection.....	60
3.1 Abstract.....	60
3.2 Introduction.....	61
3.3 Results	63
3.3.1 Saponin-induced live-cell delivery of cytoskeleton and organelle targeting antibody probes	63
3.3.2 Verifying cell membrane recovery.....	65
3.3.3 Validation of Nanogold probe conjugation and delivery.....	66
3.3.4 Preparation for electron microscopy applications	68
3.4 Methods.....	68
3.4.1 Transient cell membrane permeabilization and recovery	68
3.4.2 Live-cell delivery of exogenous probes workflow	69
3.4.3 Conjugation of fluorophore-Nanogold dual-labeled antibody probe.....	70
3.4.4 Cryo-EM	71
3.5 Conclusion and discussion.....	71
3.6 References	74
Future Directions	77
4.1 Overview	77
4.2 Future directions for SLiM.....	77
4.3 Future directions for live-cell delivery and cryo-EM labeling.....	78

List of Figures and Tables

Fig. 1.1.....	11
Table 1.1.....	13
Fig. 1.2.....	14
Fig. 1.3.....	17
Fig. 1.4.....	18
Fig. 1.5.....	21
Table 1.2.....	24
Fig. 1.6.....	25
Fig. 1.7.....	27
Fig. 2.1.....	44
Fig. 2.2.....	48
Fig. 2.3.....	51
Fig. 2.4.....	52
Fig. 2.5.....	53
Fig. 2.6.....	55
Fig. 3.1.....	66
Fig. 3.2.....	68
Fig. 3.3.....	69

Acknowledgments

It takes a village to raise a child—it truly took the whole university to grow a Ph.D.

There isn't enough time in the world to acknowledge and give thanks to every single person who has greatly impacted my experience through this program. First and foremost, I am sincerely grateful that I was able to find a principal investigator, a mentor, an adult, and a guiding hand like Professor Joshua Vaughan in the Department of Chemistry. He really helped me develop a grounded understanding of this work, challenged me to push myself to traverse beyond comfortable domains, while providing a supportive and encouraging environment for me to continue cultivating and pursuing the unknown. From all the fond memories he contributed to, including the trips out on his millionaire friend's boat, to the nights practicing music in a group band at his home, Josh did his best to make my Ph.D. journey exciting and worthwhile at every moment. I want to thank group members who had already departed at this moment, including Dr. Marcus Woodworth, who was my first graduate student mentor to teach me his ways around wet bench protocols, Dr. Chetan Poudel, who really was like a hyung (older brother) next desk space that I could shamelessly ask dumb questions to, Dr. Adilijian Ali, with whom we went through some crazy experiences, and Dr. Ziyu Guo, who had been my research buddy for the longest time and suffered through the mostly downs but also ups in both Keck and SLiM projects. Hannah and Mady, who are my defending cohort, really endured through the difficulties of research, sharing both good times and bad, but we always knew that we had each other to fall back on. Pedro, Yuka, and Wenhui, who are the next line of defense (pun intended) in this group, I am grateful for having mentored you all in your own unique ways, and I believe the group is in very capable hands moving forward. Trevor and Erica, who are the old newbies in the group, I thank you for making my last year eventful and helping to take a ton of stress off my shoulders. Special thanks to Eunjin and

Val, who really helped push the last-minute data towards the SLiM work. There are so many others in the department that I'd love to shoutout individually, but can't in detail. Firstly, my cohort members: Morgan, Crystal, Bradley, Emily, Thom, and Shardon, who I'm very proud of for the future journeys ahead of each of them. Next, my activity buddies: Yunos, Sherry, Anna, Katie, and Ruby, for all the hikes and ski trips together.

I want to end with very special thanks to my family and friends outside the department. First and foremost, my parents, who have supported me through very difficult times since the first time we immigrated to the U.S., been proud of who I am, understood this long winding path of my academic career so far, and addressed their own challenges battling health and life in a foreign country. Next, my wife, Sylvia, who was the rock that welcomed me day after day, no matter the highs and lows, to give me mental nourishment and renewed strength to move forward another day. Our pets, Zuli and Topaz, who really saw the ugly sides of me and said nothing, treated me the same, with welcoming hugs and sometimes annoying meows. Finally, my friends I made from my Alma Mater: John, Alex, and Rien, who were always inviting me to hang out and keep my head up through this journey.

Thank you everyone for making this huge impact in my life, and I mean it truly that it would not have been possible without every single one of you.

Chapter 1

Introduction

1.1 Overview of microscopy techniques

Ever since their invention and use dating back to the 16th and 17th centuries, microscopes have become an indispensable tool for revealing the natural world at small scales beyond what is resolvable by the naked eye. As technology has advanced, more innovations have been incorporated into microscope design to expand their usability in various fields, from creating chips for supercomputers to understanding biological processes. Particularly in clinical settings, microscopy is highly informative for diagnosing complex diseases, determining prognoses, and assessing treatment efficacy in microenvironments. In biological research, microscopy is an invaluable tool that allows spatial visualization of specific biological markers, validation of novel biochemical methods, and testing of cell viability and drug response, among others.

In this chapter, I will briefly review the history and advancements of select microscopy techniques tailored for specific uses, along with their advantages and disadvantages, leading to the current state of biological multiplexed imaging.

1.1.1 Important applications in research and clinical settings

In clinical settings, microscopy is commonly used to help diagnose patient health by visualizing biological samples, including blood, bone marrow, or biopsy tissue. The availability of

spatial information allows the identification of important phenotypes and the localization of biomarkers for determining disease progression and treatment response. The use of microscopy in modern biological research settings is even more diverse, both in the microscopy systems used and the chemical techniques used to extract and reveal hard-to-see biological information. In many cases, more complex, and more powerful hardware, is used compared to those in clinical settings, including, but not limited to, super-resolution, light-sheet, hyperspectral, multiphoton, and electron microscopy, each of which allows versatile biological probing suited to specific experimental purposes [1–4].

1.1.2 Comparisons between various microscopy techniques

Depending on its use, the field of microscopy can be divided into several subcategories that describe systems with varying degrees of detection capability and resolution. Broadly, microscopy techniques fall under two major categories: light microscopy and electron microscopy. Each has its own set of advantages and disadvantages, offering flexibility for users to choose the modality best suited to the requirements of a given experiment. For example, when examining larger samples (>1 mm) with multiplexed detection, light microscopy offers high throughput, ease of use, and the greatest tunability to match a variety of experimental conditions [5,6]. If the goal is to study the nanoscale spatial organization of cells in microenvironments or protein folding, then electron microscopy can reveal interactions down to the molecular or even atomic level [7–10]. Ultimately, light and electron microscopy often complement each other by balancing resolution, throughput, and multiplexing to provide a holistic view of biological systems.

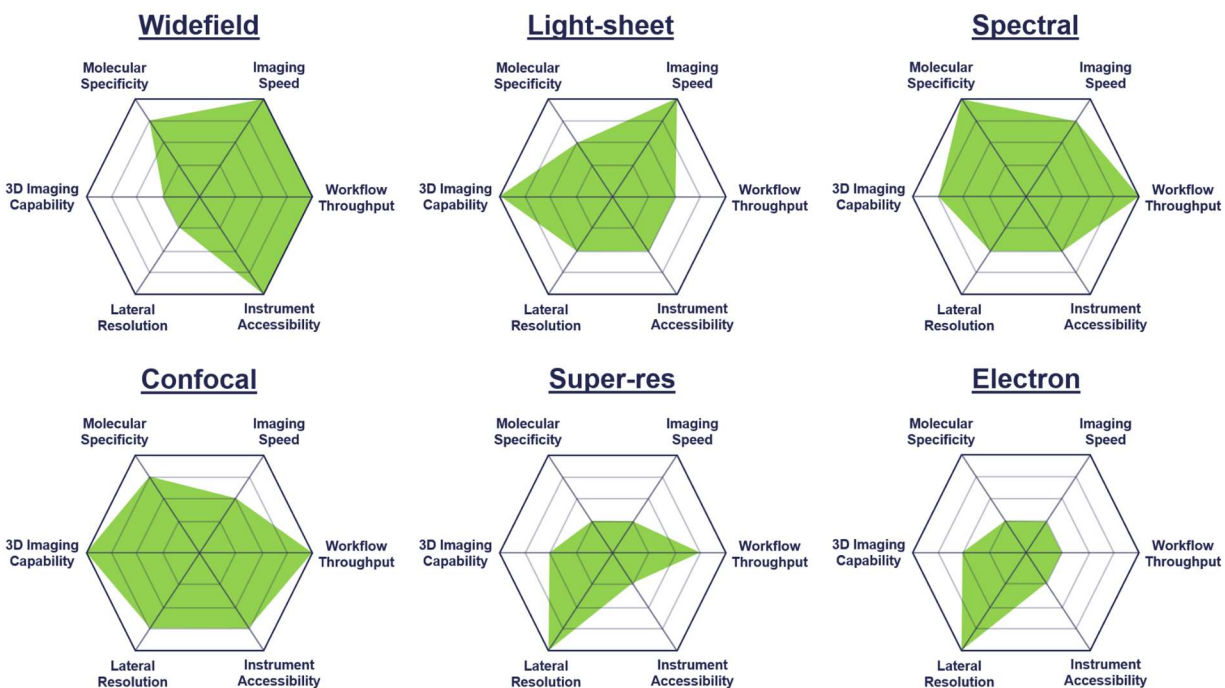


Fig. 1.1 Resolution vs. Throughput vs. Multiplexing for Microscopy Modalities.

1.1.2.1 Light microscopy

In its simplest form, microscopy originates from the use of a light source, whether a lamp, a candle, or the sun, to illuminate a sample under magnifying lenses and detect the resulting contrast-enhanced image for visualization. With the emergence of technologies to generate light sources and a critical understanding of how to manipulate light using varying geometries and refractive indices of materials, light microscopy has evolved into a versatile platform, allowing various configurations of illumination shape, wavelength, and sample detection. From a large-field-of-view, lamp-illuminated widefield microscope generally used in histology to five-laser multiplexed fluorescence confocal and even nanometer-scale illumination (e.g., point-scan confocal or STED) with single-molecule detection [11], light microscopy can support diverse applications in both research and clinical settings. With the introduction of confocal microscopy,

three-dimensional z-stack analysis can be performed on volumetric samples [12]. HILO [13] and light-sheet techniques [14,15] allow efficient optical sectioning from cells to whole mouse organs. Microscopy approaches utilizing TIRF [16], STORM [17], PALM [16], STED [18,19], and even expansion microscopy (ExM) [20] allow tens-of-nanometer-scale single-molecule analysis. Additionally, the use of bandpass filters and specialized equipment allows multiplexed, high-content imaging for imaging-based cytometry [21], transcriptomic [22,23], and live imaging applications [24,25]. One emerging field of microscopy analogous to light microscopy is Raman and stimulated Raman spectroscopy (SRS) microscopy [26]. By probing chemical content that offers invaluable insight that typical light microscopy techniques cannot provide, SRS can detect and distinguish lipids and proteins in biological samples at micron-scale resolution [27,28] based on their chemical compositions.

1.1.2.2 Electron Microscopy

In comparison to light microscopy, the limitations of electron microscopy are mainly that target specimens, typically limited to fixed cells or sections of tissues, requires rigorous sample preparation pipeline that often results in a low-throughput pipeline [1,29–31]. In addition, there are limited imaging modalities based on the electron beam for differentiating targets from the surrounding environment. In spite of these limitations, electron microscopy is the ultimate tool for spatially resolving very small objects beyond the diffraction limit of visible light. Electron microscopy is well suited to revealing details typically unresolved by the wavelengths used in analytical methods based on visible light, X-ray, IR, and Raman spectroscopy [31], and many researchers use electron microscopy to investigate the nanoscale organization of cellular organelles, chromosomes, viral morphology, and even protein structure [30,32]. To further

improve the application of electron microscopy, many efforts have been focused on increasing sample throughput through automation of sample preparation and processing, as well as innovative labeling approaches using nanoparticles or uniquely shaped tags that can be resolved and distinguished from the surrounding environment [32–34].

Technique	Resolution	Throughput	Multiplexing (routinely)	Key limitation
Optical microscopy				
Widefield	Microscale	Large field-of-view, Fast scan	3-4 channels, Sequential	No optical sectioning or out-of-focus rejection → reduced contrast in thick samples
Light-sheet	Microscale	Large field-of-view, Optical Sectioning, Fast scan	3-4 channels, Sequential	Complex alignment/calibration and sample preparation → limited accessibility, often anisotropic
Confocal	Sub-micron scale	Large field-of-view, Optical Sectioning, Medium speed scan	3-4 channels, Sequential	Limited photon efficiency due to pinhole rejection → increased photobleaching and slower acquisition
Super-resolution	Nanoscale	Point-scan, Single-molecule	1-3 channels, Sequential	Low throughput and small field-of-view → incompatible with large scale, high content imaging
Spectral (dispersive)	Microscale	Medium field-of-view, Fast scan	10+ channels, Simultaneous	Requires computational unmixing → often restricted to thin samples
Electron microscopy				
Electron	Atomic scale	Point-scan, Single-electron	1 channel	Limited multiplexing and labeling capability → requires extensive sample preparation process

Table 1.1 Comparisons of Microscopy Modalities.

1.1.3 Importance of labeling for microscopy

As briefly mentioned, there are important considerations in both light and electron microscopy and their labeling strategies for sample preparation workflows. Without labels that can identify analytes of interest within a complex cellular or tissue microenvironment, it is impossible to distinguish analytes from the background or draw comparative quantification through parallel detection that is important for spatially probing the biological system. It is not only important to “take a picture” but also to discover “what” is there. Broadly, labels or probes typically utilized in

microscopy consist of chromophores, small-molecule fluorophores, fluorescent proteins, nanoparticle fluorophores, and metal nanoparticles [24,35–37]. Using one or a combination of several labels is common depending on the target structure, its accessibility, the sample type, and the experimental goals.

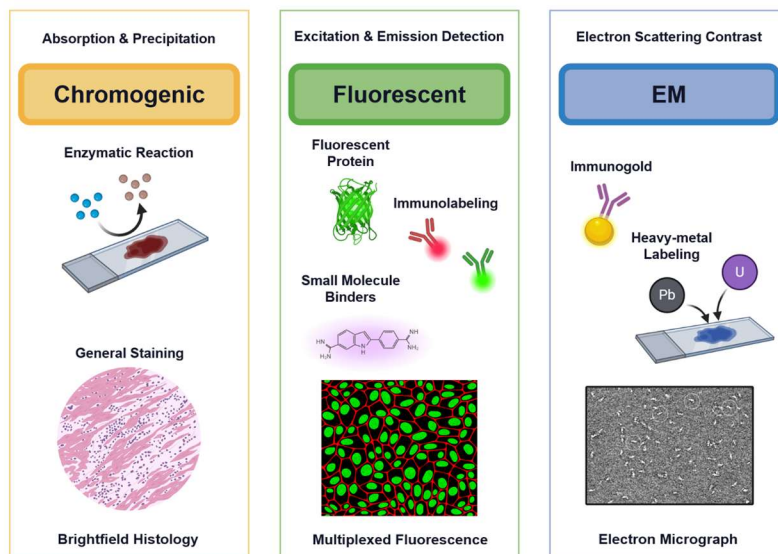


Fig. 1.2 Labeling Strategies across Imaging Modalities. Created with [BioRender.com](https://www.biorender.com).

1.1.3.1 Absorption-based and fluorescence labeling strategies

Transmitted light microscopy generally appears low-contrast and lacks distinguishable objects from the background, and often small-molecule chromophores and silver-enhanced nanoparticles are employed to introduce color and contrast to images, such as hematoxylin and eosin (H&E), melanin, azo dyes, Sudan dyes, and DAB [3,38,39]. In order to be an effective chromophore, a reagent must generate contrast and usually bind to some general structure, such as lipids, proteins, nucleic acids, and so on; in some cases, enzyme-modified binders perform the deposition of chromophores that lacks innate targeted affinity.

The availability of fluorescent probes that can specifically label target analytes has transformed the field of microscopy. While chromophores provide effective multicolor contrast through absorption-based staining, fluorescent probes allow more sensitive and selective visualization of analytes with little-to-no background. This enables clear identification of target microenvironments and more detailed spatial localization at the molecular level. As the field has evolved, new variations of dyes are constantly being synthesized to target new biomarkers and contribute to multiplexing efforts.

Small-molecule fluorophores are the bread and butter of fluorescence microscopy and can be divided into affinity-binding and functionalized variants: affinity-binding probes bind innately to a target analyte, while functionalized fluorophores require an additional targeting group to bind its analytes [2,37]. Some common affinity-binding probes include nucleic acid dyes (Hoechst, DAPI, SYBR Green), lipid dyes (DiD/DiI, BODIPY, Rhodamine), and ion-indicative dyes (MitoTracker, Fluo-4, SBFI) [35,40]. Affinity-binding or ion-indicative dyes are highly useful for specific detection of analytes and can be easily incorporated into diverse multiplexed applications in cells and tissues.

In contrast, functionalized fluorophores are small molecules that are synthesized primarily for their fluorescence properties without self-affinity for biomarkers. These fluorophores are normally synthesized with functional handles for bioconjugation by introducing reactive groups away from the main fluorescent body [40]. The use of functionalized fluorophores is limited only by the material to which they are conjugated, typically proteins. Often, affinity-binding dyes are

combined with functionalized dyes for various multiplexing applications. Immunostaining is the most popular use of affinity-binder probes, owing to antibodies' high specificity to target biomarkers. Antibody-fluorophore probes are widely available commercially and are part of an essential workflow for most biological research and clinical laboratories when measuring analyte distributions within complex biological settings. Other commonly used affinity-binder-fluorophore probes include nanobodies, lectins, aptamers, and, more recently, computationally designed minibinder conjugates [41–45].

For living specimens, fluorescent proteins are instead commonly used to label and track analytes, typically proteins [44]. Fluorescent proteins (FPs) are often incorporated into the genomic region where a protein is transcribed, commonly through transfection of a vector containing the target protein connected to an FP by a linker, or by using gene-editing tools such as CRISPR to introduce the genetic code into the host genome [36,46]. FPs are powerful for tracking native proteins in live imaging and can target most biomarkers, thereby alleviating some of the limitations of immunolabeling. However, because FPs requires genetic transformations, it can be perturbative to biological function from overexpression and adding a large sterically hindered moiety, and generally are poorer in signal strength and photon efficiency than organic fluorophores. Overall, these variations and individual constraints in probe selection motivate the need for multiplexed techniques that can parallelize visualization within a single sample [47,48].

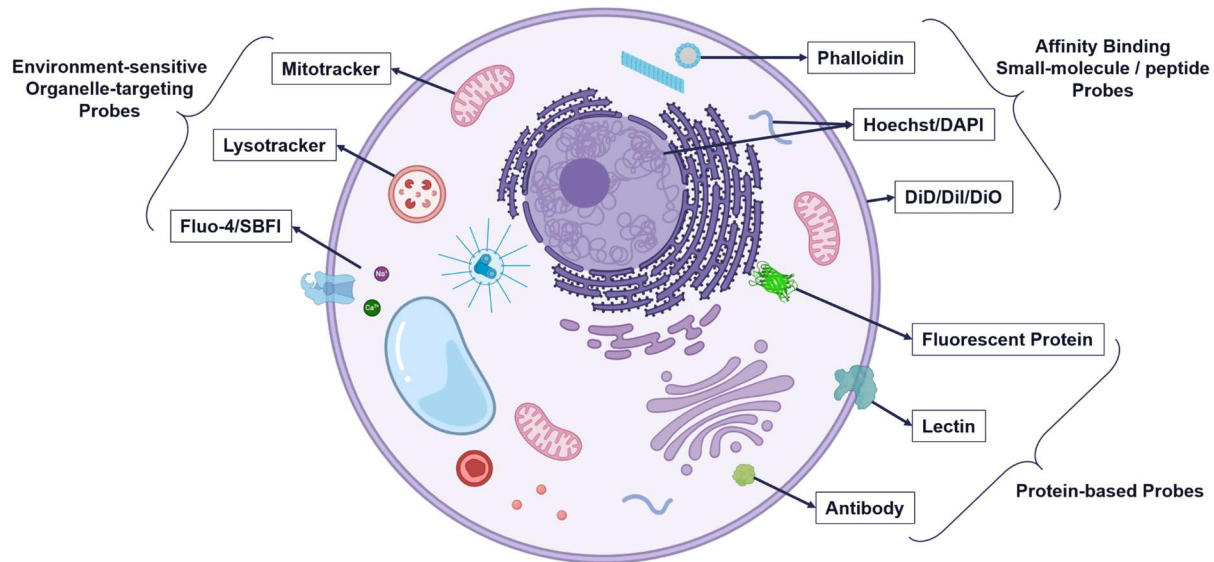


Fig. 1.3 Fluorescence Compatible Probe Types and their Typical Cellular Targets. *Dye:Target*
→ *Mitotracker:Mitochondria, Lysotracker:Lysosome, Fluo-4/SBFI:Calcium/Sodium Ions, Phalloidin:Actin, Hoechst/DAPI:DNA/RNA, DiD/Dil/DiO:Lipid, Fluorescent Protein:Endogeneously Expressed Protein, Lectin:Glycoprotein, Antibody:Protein.* Created with BioRender.com.

1.1.4 Multiplexing strategies

A central limitation of modern microscopy is detecting multiple biomarkers for correlative analysis. Multiplexing techniques address this limitation by allowing simultaneous analysis of biomarker spatial localization and enabling meaningful conclusions about their organization and function in biological systems [49]. From investigating chromatin organization in the nucleus to the cytometric distribution of cell types in tissue microenvironments, utilizing even conventional four to five colors reveal important biological implications through spatial localization. There are several key factors in multiplexing strategies to effectively separate different analytes from one another, notably in probe selection and labeling workflow.

1.1.4.1 Multicolor probe selection / Physical separation of wavelengths of light

The first of many considerations for multiplexed imaging is choosing the right set of probes. It is very common to use various combinations of the probes mentioned above to achieve multiplexed detection of biomarkers. Fluorescent probes contribute the most to this effort because their properties allow distinct excitation and emission collection of selected dyes at a given time [35]. For example, each fluorescent probe has a specific wavelength range at which it is most effectively excited [37]. Knowing their absorbance spectra allows researchers to build workflows in which a few dyes are excited at a time by using specific lasers or white light filtered by excitation filters. Similarly, each fluorophore has a different emission spectrum, which can be carefully separated using emission filters. The key to building a multicolor probe set or panel that is viable for an experiment is determining how distinctly one can excite and collect emission from one dye at a time. This mainly depends on how much the absorption and emission spectra overlap and on the types of filters used.

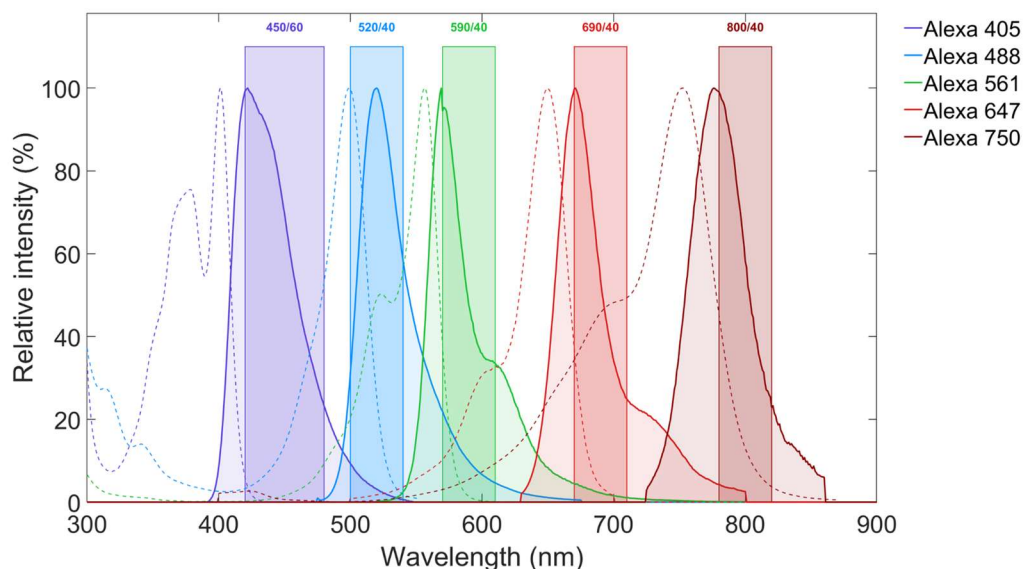


Fig. 1.4 Conventional Dyes Across the Visible and Near-Infrared Spectrum. Absorption (dashed) and emission (shaded) spectra of Alexa Fluor 405 (purple), Alexa Fluor 488 (blue), Alexa Fluor 561 (green), Alexa Fluor 647 (red), and Alexa Fluor 750 (dark red). Arbitrary emission filters are represented as 450/60, 520/40, 590/40, 690/40, and 800/40, describing the center wavelength and width of each bandpass in nanometers.

Selecting appropriate sets of fluorophores can be challenging due to several considerations. First, the imaging modality must be considered. In fluorescence microscopy, the usable fluorophores depend on what excitation sources are available and how many there are. Since fluorophores all exhibit unique absorption spectra, each is excited most effectively by lasers at specific wavelengths or by white light spanning the visible spectrum [2]. In short, the greater the number of excitation sources, the more fluorophores can be used simultaneously.

However, the second consideration limits how many of those fluorophores are actually applicable for sample labeling: spectral overlap. Typical small-molecule fluorophores have

absorption and emission spectra with widths between 30 and 150 nm, which means there are limits to the number of applicable fluorophores that can simultaneously be used without significant overlap of their spectra [35]. Although excitation multiplexing can help differentiate fluorophores, in practice the broad and overlapping absorption and emission spectra present fundamental constraints to multiplexing. Emission filters are often used to isolate fluorescence signals during detection. While emission filters are primarily required to reject excitation light, their spectral bandpass also defines the portion of emission to be collected. Commercial filters are typically within the 20–60 nm range centered at a transmittance maximum, with a finite transition region between excitation light rejection and transmission of signals [38]. Some custom filters can go below 20 nm, allowing additional fluorescent probes to fit into smaller spectral gaps [50]. In general, increasing the number of fluorophores makes signal separation increasingly difficult, and multiplexed imaging in research and clinical settings is often limited to four to five probes at a time in a single round of labeling. Researchers have found ways to extend this limit through narrower filters, fluorophores with long Stokes shifts [51], cyclic labeling techniques [49], and fluorescence lifetime [52].

Another way to improve spectral separation is to use an optical system that reorganizes light using prism or grating optics. A few limitations of filters arise from the physical constraints of filter bandwidth and the sequential nature of the acquisition workflow. Unless multiple detectors are used, the fluorescence emission rejected by the filter is typically discarded. With every acquisition at an individual filter, repeated for each fluorophore used, fluorophores are photobleached, at a rate determined by their structures, excitation light intensity and wavelength, and chemical environment [53]. This can change fluorescence properties or simply diminish the

signal-to-noise ratio. In addition, a bandpass filter captures only a limited portion of each spectrum, losing usable signal. In contrast, prism or grating optics disperse the emission spectrum onto the detector, essentially reorganizing combined wavelengths of light into a spatial dimension and thereby allowing true simultaneous detection of all excitable fluorophores in one sample [53,54]. Using this process in spectral imaging, the full emission spectrum is collected, maximizing the photon budget and preventing repeated excitation of the same fluorophore that leads to premature photobleaching.

Broadly, one limitation that stands out in the discussion above is the sequential nature of conventional multiplexed imaging techniques. In typical fluorescence microscopy, a series of signals are detected through stepwise acquisition using different excitation sources and emission filters, requiring repeated imaging of a single sample to extract spatial information across channels. During this process, the sample is subjected to photobleaching and signal inconsistencies. This raises the question whether we could utilize more than four or five fluorophores simultaneously with a single imaging modality, thereby significantly reducing the time needed for sequential acquisition. In the next section (1.2), I will go into more detail about the motivation and background for developing highly multiplexed spectral line-scan microscopy at a single excitation wavelength using polymer dots (Pdots). [

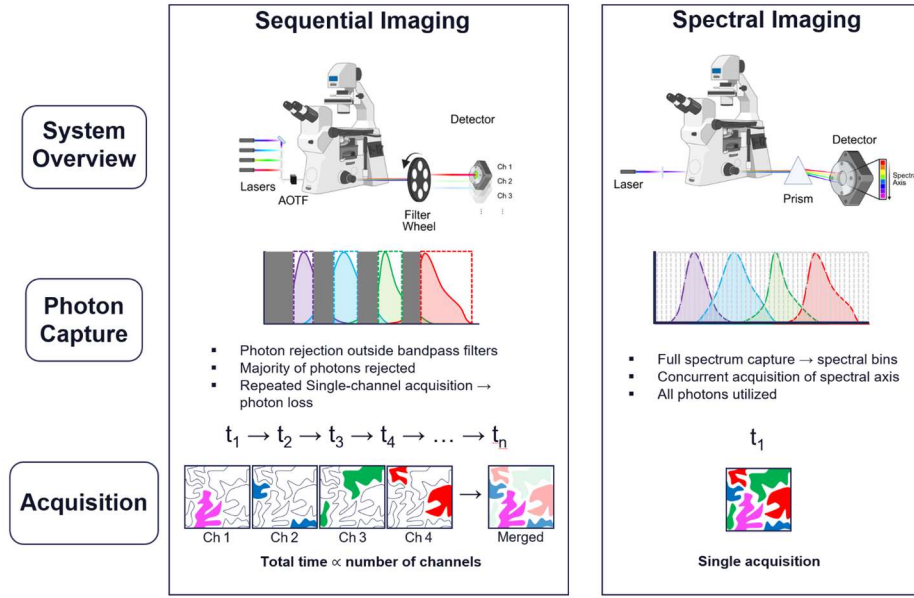


Fig. 1.5 Sequential (Filter-based) vs. Spectral (Simultaneous) Acquisition. Sequential imaging requires mechanical switching through cycling of filter wheels, presenting several limitations in photon efficiency and acquisition time. Spectral imaging simultaneously collects dispersed emission across spectral bins, retaining most photons in single acquisition.

1.1.4.2 Labeling workflow

There are a few rules of thumb when it comes to both cell and tissue labeling, whether for immunostaining, small-molecule affinity binders, or direct fluorophore conjugation to a sample. Preparing a sample for labeling usually involves three main steps—fixation, permeabilization, and blocking [38]—typically in that order, to preserve the specimen, allow transport of labeling reagents, and prevent nonspecific binding that contributes to background noise. Beyond those processes, the details of the workflow depend on the type of labeling being performed. Here, I will describe common labeling techniques and how they can be effectively combined to generate the most appropriate multicolor samples.

Immunostaining or immunolabeling, as described before, is one of the most common methods for multiplexed imaging. At the most basic level, fluorophores conjugated to biomarker-targeting primary antibodies allow visualization as direct immunofluorescence or, when conjugated to secondary antibodies targeting primary, as an indirect immunofluorescence [41,55]. In terms of precision, direct immunolabeling methods minimize the distance between target and signal; however, indirect immunolabeling offers signal amplification at a lower overall cost and ease of preparation [56]. For conventional microscopy, indirect immunolabeling is typically capable of achieving two to three imaging channels. However, if one wishes to go beyond typical two to three-color imaging, it becomes increasingly challenging to find combinations of primary and secondary antibodies that avoid unwanted cross-reactivity. Direct immunolabeling has no such limitation on using antibodies from the same host simultaneously, since fluorescence is measured directly from the primary antibodies, but the conjugation process is usually labor-intensive and expensive to produce at scale [56].

To address this issue, a range of strategies have been employed that use antibody removal or fluorophore inactivation in cyclic workflows, allowing sequential rounds of biomarker labeling and visualization of ~3 markers at a time to build up to 10-100 total channels [57]. Instead of antibody conjugates, oligonucleotide-conjugated antibodies are also commonly explored for cyclic immunolabeling, combining oligonucleotide hybridization with immunostaining, where fluorophores are instead conjugated to the 5' end of oligonucleotides [58,59]. Using these cyclic immunostaining techniques, researchers can achieve greater than 65-color images [60], limited mainly by sample retention, processing time, and the number of available markers.

1.1.4.3 Finding the balance between highly multiplexed images and fast turnaround time

By implementing combinations of these multiplexed techniques, researchers can ideally achieve more than enough colors for meaningful biological interpretation in both clinical and research settings for both diagnosis and hypothesis testing [61]. Highly multiplexed imaging beyond the typical number of channels in a single round of labeling can inform the important tumor microenvironments and the response to immunotherapy [62] and describe structural organization at single-cell resolution of vital organs to categorize and describe local and global relationships that define their function [63,64].

With combinatorial tactics in which barcodes are introduced, the number of unique signals can, in principle, be extended to extremely high levels [65–67]. For example, approaches using fluorescent proteins or DNA-based barcodes enable robust identification of cell-level information for live imaging and transcriptomics, while emerging techniques such as laser particles suggest the possibility of identifying 10^5 - 10^6 unique optical signatures. However, despite these advancements, there still remains a practical balance between the number of multiplexed targets and the time required for analysis. Often, it is more important to process a sample quickly for rapid diagnosis than to spend days to weeks generating highly multiplexed images [61,68,69]. So how do we achieve optimal multiplexed imaging within a short time while still drawing meaningful conclusions? The answer will clearly vary depending on the type of sample, the goal of the study, and our understanding of the relationships between biomarkers and biological implications.

Technique	Multiplexing capacity	Acquisition/processing time	Biological perturbation	Instrument complexity	Multiplexing modality	Strength	Weakness
Traditional filter-based sequential imaging	Moderate (4-5 channels)	Moderate to High (Sequential acquisition per channel)	Low	Moderate	Spectral separation via bandpass filters	Conventional and easy to implement	Sequential acquisition causes photobleaching and discards out-of-band photons
Spectral imaging	High (10+ channels)	Low (Simultaneous acquisition of all channels)	Low	Moderate	Simultaneous acquisition across spectrum + unmixing	Simultaneous detection with high photon efficiency	Complex system requiring alignment and spectral calibration
Multi-cyclic fluorescence labeling	Very High (50+ channels)	High (Sequential acquisition + cyclic immunolabeling)	High (Iterative probe removal and labeling)	High	Temporal separation via iterations	Ultra-high multiplexing via iterative labeling cycles	Extensive sample preparation and long acquisition time. Sometimes perturbative for denaturation-based antibody stripping

Table 1.2 Comparisons between Multiplexed Strategies.

1.2 Highly multiplexed spectral line-scan microscopy at single-excitation wavelength

1.2.1 Limitations of widely conventional sequential labeling and imaging technique

Multiplexed techniques center around the idea of maximizing the number of usable fluorescence signals while minimizing crosstalk and sample degradation related to warping, cell/antigen loss, and bleaching [69]. For this reason, the process of multiplexed imaging is inherently sequential, from sample preparation, such as cyclic immunostaining, to signal separation, such as filter-based imaging. Specifically, the three main motivations for improving multiplexed techniques with spectral imaging are reducing time to label, reducing time to image, and improving the quality of the resulting signal.

Cyclic labeling methods to achieve beyond five-color imaging require additional time spent deactivating or quenching existing fluorophores or removing antibodies and relabeling with new

colored probes. During this time, it is common to observe feature distortion, biomarker depletion, and structural perturbation following cyclic labeling [69]. In addition, depending on sample size and thickness, the required number of reagents and diffusion time can vary greatly, with uneven binding characteristics from the surface to the interior of the sample.

Time to image is largely dependent on the microscope and software available on a given system, but conceptually the acquisition is also performed sequentially, with mechanical switching between lasers, dichroic mirrors, and filter options. As expected, each fluorescent probe requires a unique combination of these optical controls, which adds time to the acquisition program [1]. In addition, if imaging at multiple positions is required, then stage movement time is increased accordingly. These mechanical constraints can sometimes be addressed through efficient filter sequencing to find a system-dependent optimal pattern; however, sequential imaging affects not only acquisition time but also the images themselves. At each imaging step, filter-rejected emissions are effectively discarded, leading to inefficient utilization of the photon budget.

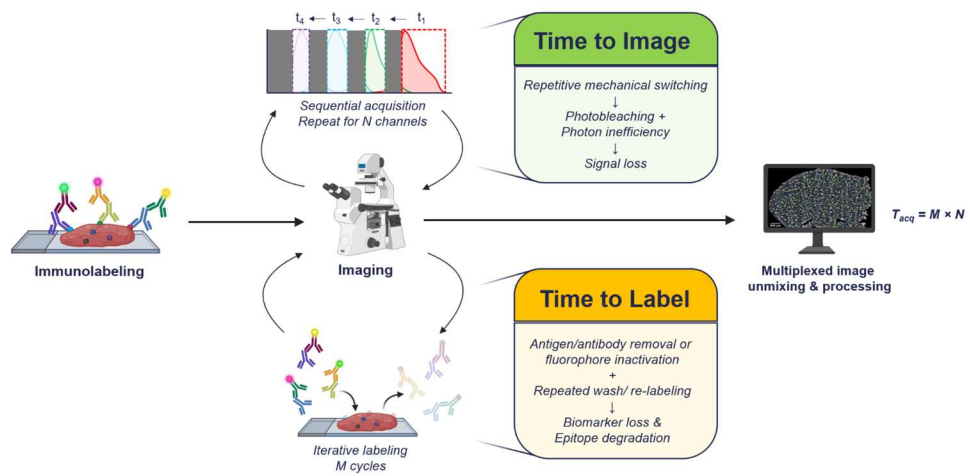


Fig. 1.7 Bottlenecks for Conventional Sequential Workflow.

Both times to label and image are important factors when designing a multiplexed imaging pipeline. There are multiple strategies aimed at resolving fluorophore emission overlap and mechanical switching. Here, I will discuss two specific techniques that I use in combination to improve multiplexed imaging by enabling simultaneous fluorophore detection, eliminating mechanical switching, and reducing photobleaching.

1.2.2 Leveraging emergence of polymer dots

Polymer dots, or Pdots, are semiconducting nanoparticles composed of semiconducting polymers sequestered into a compact “ball” with sizes typically ranging from 5 to 20 nm [70]. The unique composition of Pdots allows them to be bright, owing to their large absorption cross-section, and have long Stokes shifts that can be spectrally tuned from 20 to 400 nm from the absorption maximum. Some small molecule fluorophores are available with a long Stokes shift, but these tend to have a very large emission spectral width that hinders efforts at multiplexing [71–73]. Tandem dyes are also sometimes used as probes for creating a large Stokes shift without a significantly broadened fluorescence bandwidth, such as Astral Leap, NovaFluor, or ATTO LS dyes [74,75]. These dyes are great strategies for retaining high quantum yield and generating dyes with spectrally tunable Stokes shifts; however, they usually display broad absorption spectra, which is disadvantageous for excitation multiplexing, and often suffer inconsistencies between lots from variability in conjugation. Quantum dots, or Qdots, are also considered as options for multiplexed probe panels because of their narrow emissions and large Stokes shifts [76]; however, due to their inorganic composition, their absorption spectra tend to broadly span the UV-to-visible region, making excitation multiplexing extremely difficult because of cross-excitation, along with lower-than-typical absorption efficiency at a single wavelength. In contrast to both tandem dyes

and Qdots, Pdots are usually brighter and can be designed to be simultaneously excited at a single wavelength by incorporating absorber molecules tuned for specific excitation wavelengths. By leveraging this probe design, multiple Pdot series that simultaneously excite differently emitting molecules at various wavelengths can be synthesized. For example, in the 405 nm excitation series used in this work, there are eleven possible Pdots with unique, narrow emission profiles across the visible and near-infrared spectrum (400-800 nm).

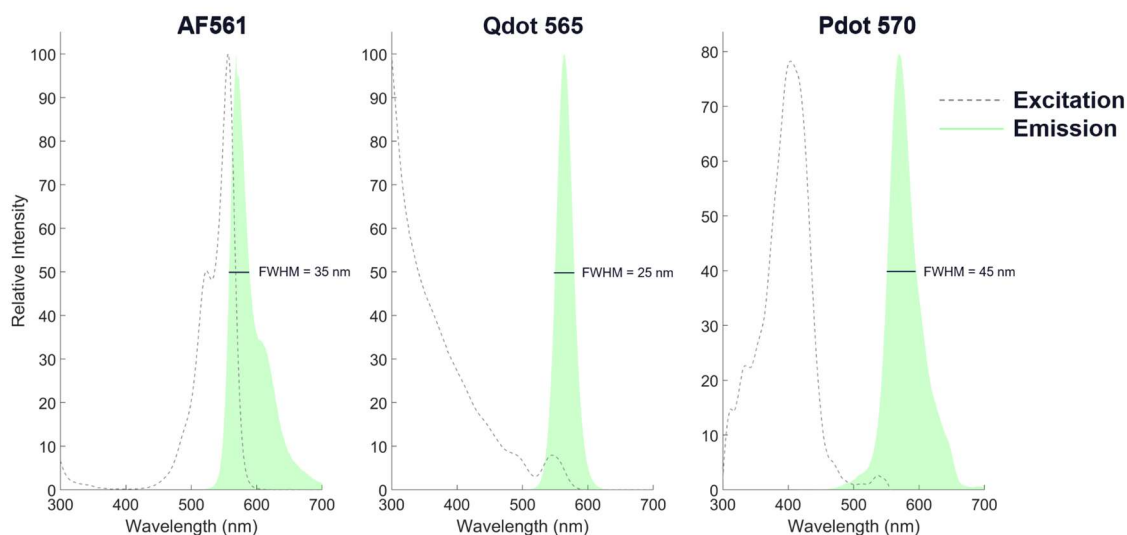


Fig. 1.8 Fluorescence Excitation (dashed) and Emission (shaded) Spectra for AF561, Qdot 565, and Pdot 570. All probes have a similar emission bandwidth (25-45nm).

1.3 Live-cell labeling for cryogenic electron microscopy

1.3.1 Limitations of heavy-metal labeling technique

As the name suggests, electron microscopy utilizes electrons to visualize the absorbance, scattering, and transmittance of materials within biological samples. Conventionally, electron

microscopy utilizes heavy-metal labels such as uranyl acetate, osmium tetroxide, lead citrate, and potassium permanganate for transmission electron microscopy (TEM) to enhance sample contrast by creating electron-dense areas for additional scattering or absorption during imaging [31]. Similar to immunofluorescence in fluorescence microscopy, immunogold techniques using colloidal gold or nanogold [77] are commonly employed to perform targeted labeling in biological samples, where contrast is specified at localized regions or analytes of interest.

Normally, intracellular labeling methods for electron microscopy are only compatible with chemically fixed and embedded samples due to the necessary cell membrane permeabilization step. However, perturbations induced by chemical fixation and detergent permeabilization often disrupt the native nanoenvironment, destroying structural integrity and distorting morphology [78–80]. Cryogenic electron microscopy (Cryo-EM) is superior to chemical fixation and embedding methods because it can retain native structures by flash-freezing samples below the melting point of ethane and capturing structural states in vitreous ice [81]. However, because of this workflow, it is difficult to perform deep, penetrative labeling after cryofixation without significant effort, as with cryo-freeze substitution, for intracellular applications [82,83]. As such, multiplexed imaging for Cryo-EM with heavy-metal labeling has remained largely limited to surface-level targets.

1.3.2 Motivation for live-cell delivery of exogenous probes

Labeling cryofixed samples through approaches like cryo-freeze substitution that involve technically demanding workflows that involve long processing times, low throughput, and the potential risk of structural alterations. One alternative way to introduce exogenous probes while alleviating these limitations is live-cell delivery. Live-cell delivery strategies have long been

investigated for endogenous expression and engineering of proteins and DNA, drug delivery, as well as labeling [84–88], but generally they require specialized instrumentation incompatible with EM workflows using cultured cells on EM grids, or they have extremely low throughput. Therefore, to realize live-cell delivery for EM applications, another strategy must be devised that allows direct delivery to cells on EM grids while enabling rapid recovery prior to cryofixation to retain native structures.

To this end, I devised a method, along with UW Bioengineering and Biochemistry groups (Gao, Baker, Kollman, King), to introduce exogenous labels to live cells prior to the Cryo-EM workflow. By utilizing a mild chemical detergent called saponin, I can transiently permeabilize the cell membrane to introduce fluorophore-nanogold dual-labeled antibody probes, then recover the membrane prior to cryofixation. The entire permeabilization, labeling, and fixation pipeline occurs within 30 minutes, labeling intracellular targets while retaining live-cell morphology. I will discuss this in more detail in Section 3 of this dissertation.

1.4 Summary

Multiplexed imaging is currently at the forefront of biological research and clinical applications for understanding complex tissue and cellular environments and aiding diagnosis in medical science. By investigating and streamlining emerging techniques discussed here, such as 11-channel spectral imaging and live-cell delivery of exogenous labels for Cryo-EM, we can advance the use of existing instruments toward the next generation of multiplexed applications. There are many considerations involved in these techniques, including imaging systems, optics,

fluorescent probe selection, and image analysis, which I will discuss in detail in the following chapters.

1.5 References

1. D. B. Murphy and M. W. Davidson, *Fundamentals of Light Microscopy and Electronic Imaging*, 2nd ed (Wiley-Blackwell, 2013).
2. J. W. Lichtman and J.-A. Conchello, "Fluorescence microscopy," *Nat. Methods* **2**(12), 910–919 (2005).
3. C. R. Taylor and R. M. Levenson, "Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II," *Histopathology* **49**(4), 411–424 (2006).
4. L. Pantanowitz, P. N. Valenstein, A. J. Evans, K. J. Kaplan, J. D. Pfeifer, D. C. Wilbur, L. C. Collins, and T. J. Colgan, "Review of the current state of whole slide imaging in pathology," *J. Pathol. Inform.* **2**(1), 36 (2011).
5. J. B. Pawley, ed., *Handbook of Biological Confocal Microscopy*, 3rd ed (Springer, 2006).
6. A. Diaspro, F. Federici, and M. Robello, "Influence of refractive-index mismatch in high-resolution three-dimensional confocal microscopy," *Appl. Opt.* **41**(4), 685 (2002).
7. Y.-T. Liu, C.-L. Tao, P.-M. Lau, Z. H. Zhou, and G.-Q. Bi, "Postsynaptic protein organization revealed by electron microscopy," *Curr. Opin. Struct. Biol.* **54**, 152–160 (2019).
8. V. Lučić, R. Fernández-Busnadiego, U. Laugks, and W. Baumeister, "Hierarchical detection and analysis of macromolecular complexes in cryo-electron tomograms using Pyto software," *J. Struct. Biol.* **196**(3), 503–514 (2016).
9. B. A. Barad, M. Medina, D. Fuentes, R. L. Wiseman, and D. A. Grotjahn, "Quantifying organellar ultrastructure in cryo-electron tomography using a surface morphometrics pipeline," *J. Cell Biol.* **222**(4), e202204093 (2023).
10. M. Shekhar, G. Terashi, C. Gupta, D. Sarkar, G. Debussche, N. J. Sisco, J. Nguyen, A. Mondal, J. Vant, P. Fromme, W. D. Van Horn, E. Tajkhorshid, D. Kihara, K. Dill, A. Perez, and A. Singharoy, "CryoFold: Determining protein structures and data-guided ensembles from cryo-EM density maps," *Matter* **4**(10), 3195–3216 (2021).
11. S. W. Hell, "Far-field optical nanoscopy," *Science* **316**(5828), 1153–1158 (2007).
12. T. Wilson, "Optical sectioning in fluorescence microscopy: OPTICAL SECTIONING IN FLUORESCENCE MICROSCOPY," *J. Microsc.* **242**(2), 111–116 (2011).

13. M. Tokunaga, N. Imamoto, and K. Sakata-Sogawa, "Highly inclined thin illumination enables clear single-molecule imaging in cells," *Nat. Methods* **5**(2), 159–161 (2008).
14. B.-C. Chen, W. R. Legant, K. Wang, L. Shao, D. E. Milkie, M. W. Davidson, C. Janetopoulos, X. S. Wu, J. A. Hammer, Z. Liu, B. P. English, Y. Mimori-Kiyosue, D. P. Romero, A. T. Ritter, J. Lippincott-Schwartz, L. Fritz-Laylin, R. D. Mullins, D. M. Mitchell, J. N. Bembenek, A.-C. Reymann, R. Bohme, S. W. Grill, J. T. Wang, G. Seydoux, U. S. Tulu, D. P. Kiehart, and E. Betzig, "Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution," *Science* **346**(6208), 1257998–1257998 (2014).
15. C. Poudel, D. Brenes, W. Xie, Y. Guan, P. Vasquez, N. Mahadevan, E. Konatham, N. Fan, Z. Guo, N. J. Porter, M. K. Wong, M. M. Martinez, R. M. Sandoval, K. W. Dunn, P. C. Dagher, D. G. Eng, S. J. Shankland, J. T. C. Liu, and J. C. Vaughan, "High-throughput, organ-scale 3D tubule tracking using TubuleMAP," (2026).
16. E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacio, M. W. Davidson, J. Lippincott-Schwartz, and H. F. Hess, "Imaging Intracellular Fluorescent Proteins at Nanometer Resolution," *Science* **313**(5793), 1642–1645 (2006).
17. M. J. Rust, M. Bates, and X. Zhuang, "Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM)," *Nat. Methods* **3**(10), 793–796 (2006).
18. S. W. Hell and J. Wichmann, "Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy," *Opt. Lett.* **19**(11), 780–782 (1994).
19. S. W. Hell, "Far-Field Optical Nanoscopy," *Science* **316**(5828), 1153–1158 (2007).
20. F. Chen, P. W. Tillberg, and E. S. Boyden, "Expansion microscopy," *Science* **347**(6221), 543–548 (2015).
21. J. Y. Han, O. D. Rahmanian, E. L. Kendall, N. Fleming, and D. L. DeVoe, "Screw-actuated displacement micropumps for thermoplastic microfluidics," *Lab. Chip* **16**(20), 3940–3946 (2016).
22. Y. Lee, M. Lee, Y. Shin, K. Kim, and T. Kim, "Spatial Omics in Clinical Research: A Comprehensive Review of Technologies and Guidelines for Applications," *Int. J. Mol. Sci.* **26**(9), 3949 (2025).
23. V. Gandin, J. Kim, L.-Z. Yang, Y. Lian, T. Kawase, A. Hu, K. Rokicki, G. Fleishman, P. Tillberg, A. A. Castrejon, C. Stringer, S. Preibisch, and Z. J. Liu, "Deep-tissue transcriptomics and subcellular imaging at high spatial resolution," *Science* **388**(6744), eadq2084 (2025).
24. M. Chalfie, Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher, "Green fluorescent protein as a marker for gene expression," *Science* **263**, 802–805 (1994).
25. A. P. Cuny, F. P. Schlottmann, J. C. Ewald, S. Pelet, and K. M. Schmoller, "Live cell microscopy: From image to insight," *Biophys. Rev.* **3**(2), 021302 (2022).

26. K. Hamada, K. Fujita, N. I. Smith, M. Kobayashi, Y. Inouye, and S. Kawata, "Raman microscopy for dynamic molecular imaging of living cells," *J. Biomed. Opt.* **13**(04), 1 (2008).
27. M. C. Wang, W. Min, C. W. Freudiger, G. Ruvkun, and X. S. Xie, "RNAi screening for fat regulatory genes with SRS microscopy," *Nat. Methods* **8**(2), 135–138 (2011).
28. E. L. Dunnington, K. W. Chiu, B. S. Wong, A. Chales-Antonio, V. Pang, Y. Wu, and D. Fu, "Label-Free Longitudinal Imaging of Single Cell Drug Response with a 3D-Printed Cell Culture Platform," *Anal. Chem.* **97**(47), 26118–26129 (2025).
29. J. Dubochet, M. Adrian, J.-J. Chang, J.-C. Homo, J. Lepault, A. W. McDowell, and P. Schultz, "Cryo-electron microscopy of vitrified specimens," *Q. Rev. Biophys.* **21**(2), 129–228 (1988).
30. E. Nogales and S. H. W. Scheres, "Cryo-EM: A Unique Tool for the Visualization of Macromolecular Complexity," *Mol. Cell* **58**(4), 677–689 (2015).
31. J. Kuo, ed., *Electron Microscopy: Methods and Protocols*, Methods in Molecular Biology (Humana Press, 2014), **1117**.
32. Y. Hsia, J. B. Bale, S. Gonen, D. Shi, W. Sheffler, K. K. Fong, U. Nattermann, C. Xu, P.-S. Huang, R. Ravichandran, S. Yi, T. N. Davis, T. Gonen, N. P. King, and D. Baker, "Design of a hyperstable 60-subunit protein icosahedron," *Nature* **535**(7610), 136–139 (2016).
33. T. M. Mayhew, C. Mühlfeld, D. Vanhecke, and M. Ochs, "A review of recent methods for efficiently quantifying immunogold and other nanoparticles using TEM sections through cells, tissues and organs," *Ann. Anat. - Anat. Anz.* **191**(2), 153–170 (2009).
34. I. Schmidt-Krey and Y. Cheng, eds., *Electron Crystallography of Soluble and Membrane Proteins: Methods and Protocols*, Methods in Molecular Biology (Humana Press, 2013), **955**.
35. W. T. Mason, *Fluorescent and Luminescent Probes for Biological Activity: A Practical Guide to Technology for Quantitative Real-Time Analysis*, Biological Techniques Series (Elsevier Science, 1999).
36. B. N. G. Giepmans, S. R. Adams, M. H. Ellisman, and R. Y. Tsien, "The Fluorescent Toolbox for Assessing Protein Location and Function," *Science* **312**(5771), 217–224 (2006).
37. L. D. Lavis and R. T. Raines, "Bright Ideas for Chemical Biology," *ACS Chem. Biol.* **3**(3), 142–155 (2008).
38. K. S. Suvarna, C. Layton, and J. D. Bancroft, eds., *Theory and Practice of Histological Techniques*, 7. ed (Elsevier Churchill Livingstone, 2013).
39. J. A. Ramos-Vara and M. A. Miller, "When Tissue Antigens and Antibodies Get Along: Revisiting the Technical Aspects of Immunohistochemistry—The Red, Brown, and Blue Technique," *Vet. Pathol.* **51**(1), 42–87 (2014).
40. L. D. Lavis, "Teaching Old Dyes New Tricks: Biological Probes Built from Fluoresceins and Rhodamines," *Annu. Rev. Biochem.* **86**(1), 825–843 (2017).

41. C. B. Saper, "A Guide to the Perplexed on the Specificity of Antibodies," *J. Histochem. Cytochem.* **57**(1), 1–5 (2009).
42. T. Pleiner, M. Bates, S. Trakhanov, C.-T. Lee, J. E. Schliep, H. Chug, M. Böhning, H. Stark, H. Urlaub, and D. Görlich, "Nanobodies: site-specific labeling for super-resolution imaging, rapid epitope-mapping and native protein complex isolation," *eLife* **4**, e11349 (2015).
43. A. D. Keefe, S. Pai, and A. Ellington, "Aptamers as therapeutics," *Nat. Rev. Drug Discov.* **9**(7), 537–550 (2010).
44. R. Y. Tsien, "The green fluorescent protein," *Annu. Rev. Biochem.* **67**(1), 509–544 (1998).
45. A. Chevalier, D.-A. Silva, G. J. Rocklin, D. R. Hicks, R. Vergara, P. Murapa, S. M. Bernard, L. Zhang, K.-H. Lam, G. Yao, C. D. Bahl, S.-I. Miyashita, I. Goresnik, J. T. Fuller, M. T. Koday, C. M. Jenkins, T. Colvin, L. Carter, A. Bohn, C. M. Bryan, D. A. Fernández-Velasco, L. Stewart, M. Dong, X. Huang, R. Jin, I. A. Wilson, D. H. Fuller, and D. Baker, "Massively parallel de novo protein design for targeted therapeutics," *Nature* **550**(7674), 74–79 (2017).
46. N. C. Shaner, P. A. Steinbach, and R. Y. Tsien, "A guide to choosing fluorescent proteins," *Nat. Methods* **2**(12), 905–909 (2005).
47. M. Streit, M. Budiarta, M. Jungblut, and G. Beliu, "Fluorescent labeling strategies for molecular bioimaging," *Biophys. Rep.* **5**(1), 100200 (2025).
48. L. M. Costantini and E. L. Snapp, "Fluorescent Proteins in Cellular Organelles: Serious Pitfalls and Some Solutions," *DNA Cell Biol.* **32**(11), 622–627 (2013).
49. J. W. Hickey, E. K. Neumann, A. J. Radtke, J. M. Camarillo, R. T. Beuschel, A. Albanese, E. McDonough, J. Hatler, A. E. Wiblin, J. Fisher, J. Croteau, E. C. Small, A. Sood, R. M. Caprioli, R. M. Angelo, G. P. Nolan, K. Chung, S. M. Hewitt, R. N. Germain, J. M. Spraggins, E. Lundberg, M. P. Snyder, N. L. Kelleher, and S. K. Saka, "Spatial mapping of protein composition and tissue organization: a primer for multiplexed antibody-based imaging," *Nat. Methods* (2021).
50. P. Favreau, C. Hernandez, A. S. Lindsey, D. F. Alvarez, T. Rich, P. Prabhat, and S. J. Leavesley, "Thin-film tunable filters for hyperspectral fluorescence microscopy," *J. Biomed. Opt.* **19**(1), 011017 (2013).
51. J. Yang, Z. Xu, L. Yu, B. Wang, R. Hu, J. Tang, J. Lv, H. Xiao, X. Tan, G. Wang, J. Li, Y. Liu, P. Shao, and B. Zhang, "Organic Fluorophores with Large Stokes Shift for the Visualization of Rapid Protein and Nucleic Acid Assays," *Angew. Chem. Int. Ed.* **63**(17), e202318800 (2024).
52. F. Lin, C. Zhang, Z. Huang, Y. Wang, M. Yi, J. Li, X. Weng, Y. Chen, P. Lai, and J. Qu, "Advances in fluorescence lifetime imaging microscopy: Techniques and biomedical applications," *Appl. Phys. Rev.* **13**(1), 011303 (2026).
53. Y. Garini, I. T. Young, and G. McNamara, "Spectral imaging: Principles and applications," *Cytometry A* **69A**(8), 735–747 (2006).

54. M. E. Dickinson, G. Bearman, S. Tille, R. Lansford, and S. E. Fraser, "Multi-Spectral Imaging and Linear Unmixing Add a Whole New Dimension to Laser Scanning Fluorescence Microscopy," *BioTechniques* **31**(6), 1272–1278 (2001).
55. E. C. Stack, C. Wang, K. A. Roman, and C. C. Hoyt, "Multiplexed immunohistochemistry, imaging, and quantitation: A review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis," *Methods* **70**(1), 46–58 (2014).
56. J. A. Ramos-Vara, "Technical Aspects of Immunohistochemistry," *Vet. Pathol.* **42**(4), 405–426 (2005).
57. J.-R. Lin, M. Fallahi-Sichani, and P. K. Sorger, "Highly multiplexed imaging of single cells using a high-throughput cyclic immunofluorescence method," *Nat. Commun.* **6**, 8390 (2015).
58. Y. Goltsev, N. Samusik, J. Kennedy-Darling, S. Bhate, M. Hale, G. Vazquez, S. Black, and G. P. Nolan, "Deep Profiling of Mouse Splenic Architecture with CODEX Multiplexed Imaging," *Cell* **174**(4), 968-981.e15 (2018).
59. S. K. Saka, Y. Wang, J. Y. Kishi, A. Zhu, Y. Zeng, W. Xie, K. Kirli, C. Yapp, M. Cicconet, B. J. Beliveau, S. W. Lapan, S. Yin, M. Lin, E. S. Boyden, P. S. Kaeser, G. Pihan, G. M. Church, and P. Yin, "Immuno-SABER enables highly multiplexed and amplified protein imaging in tissues," *Nat. Biotechnol.* 1–11 (2019).
60. A. J. Radtke, E. Kandov, B. Lowekamp, E. Speranza, C. J. Chu, A. Gola, N. Thakur, R. Shih, L. Yao, Z. R. Yaniv, R. T. Beuschel, J. Kabat, J. Croteau, J. Davis, J. M. Hernandez, and R. N. Germain, "IBEX: A versatile multiplex optical imaging approach for deep phenotyping and spatial analysis of cells in complex tissues," *Proc. Natl. Acad. Sci.* **117**(52), 33455–33465 (2020).
61. M. Angelo, S. C. Bendall, R. Finck, M. B. Hale, C. Hitzman, A. D. Borowsky, R. M. Levenson, J. B. Lowe, S. D. Liu, S. Zhao, Y. Natkunam, and G. P. Nolan, "Multiplexed ion beam imaging of human breast tumors," *Nat. Med.* **20**(4), 436–442 (2014).
62. X. Xiao, Q. Guo, C. Cui, Y. Lin, L. Zhang, X. Ding, Q. Li, M. Wang, W. Yang, Y. Kong, and R. Yu, "Multiplexed imaging mass cytometry reveals distinct tumor-immune microenvironments linked to immunotherapy responses in melanoma," *Commun. Med.* **2**(1), 131 (2022).
63. J. W. Hickey, W. R. Becker, S. A. Nevins, A. Horning, A. E. Perez, C. Zhu, B. Zhu, B. Wei, R. Chiu, D. C. Chen, D. L. Cotter, E. D. Esplin, A. K. Weimer, C. Caraccio, V. Venkataramanan, C. M. Schürch, S. Black, M. Brbić, K. Cao, S. Chen, W. Zhang, E. Monte, N. R. Zhang, Z. Ma, J. Leskovec, Z. Zhang, S. Lin, T. Longacre, S. K. Plevritis, Y. Lin, G. P. Nolan, W. J. Greenleaf, and M. Snyder, "Organization of the human intestine at single-cell resolution," *Nature* **619**(7970), 572–584 (2023).
64. A. Ali, Z. Liu, K. Ye, Y. Guan, S. Chen, T. Liu, Z. Guo, M. K. Wong, P. Vasquez, C. Poudel, B. C. Mustonen, D. G. Eng, J. W. Pippin, S. J. Shankland, S. Wang, and J. C.

- Vaughan, "Super-resolved optical imaging, reconstruction, and spatial analysis of whole mouse glomeruli via the Glomerulus Mapping and Analysis Pipeline," *Kidney Int.* (n.d.).
65. T. Kaufman, E. Nitzan, N. Firestein, M. B. Ginzberg, S. Iyengar, N. Patel, R. Ben-Hamo, Z. Porat, J. Hunter, A. Hilfinger, V. Rotter, R. Kafri, and R. Straussman, "Visual barcodes for clonal-multiplexing of live microscopy-based assays," *Nat. Commun.* **13**(1), 2725 (2022).
 66. X. Fu, L. Sun, R. Dong, J. Y. Chen, R. Silakit, L. F. Condon, Y. Lin, S. Lin, R. D. Palmiter, and L. Gu, "Polony gels enable amplifiable DNA stamping and spatial transcriptomics of chronic pain," *Cell* **185**(24), 4621-4633.e17 (2022).
 67. N. Martino, H. Yan, G. Abbott, M. Fahlberg, S. Forward, K.-H. Kim, Y. Wu, H. Zhu, S. J. J. Kwok, and S.-H. Yun, "Large-scale combinatorial optical barcoding of cells with laser particles," *Light Sci. Appl.* **14**(1), 148 (2025).
 68. J. Einhaus, A. Rochwarger, S. Mattern, B. Gaudillière, and C. M. Schürch, "High-multiplex tissue imaging in routine pathology—are we there yet?," *Virchows Arch.* **482**(5), 801–812 (2023).
 69. C. Zhao and R. N. Germain, "Multiplex imaging in immuno-oncology," *J. Immunother. Cancer* **11**(10), e006923 (2023).
 70. C. Wu and D. T. Chiu, "Highly Fluorescent Semiconducting Polymer Dots for Biology and Medicine," *Angew. Chem. Int. Ed.* **52**(11), 3086–3109 (2013).
 71. M. H. W. Stopel, C. Blum, and V. Subramaniam, "Excitation Spectra and Stokes Shift Measurements of Single Organic Dyes at Room Temperature," *J. Phys. Chem. Lett.* **5**(18), 3259–3264 (2014).
 72. P. Horváth, P. Šebej, T. Šolomek, and P. Klán, "Small-Molecule Fluorophores with Large Stokes Shifts: 9-Iminopyronin Analogues as Clickable Tags," *J. Org. Chem.* **80**(3), 1299–1311 (2015).
 73. I. Likhotkin, R. Lincoln, M. L. Bossi, A. N. Butkevich, and S. W. Hell, "Photoactivatable Large Stokes Shift Fluorophores for Multicolor Nanoscopy," *J. Am. Chem. Soc.* **145**(3), 1530–1534 (2023).
 74. J. B. Woehrstein, M. T. Strauss, L. L. Ong, B. Wei, D. Y. Zhang, R. Jungmann, and P. Yin, "Sub-100-nm metafluorophores with digitally tunable optical properties self-assembled from DNA," *Sci. Adv.* **3**(6), e1602128 (2017).
 75. Y. Seong, D. X. Nguyen, Y. Wu, A. Thakur, F. Harding, and T. A. Nguyen, "Novel PE and APC tandems: Additional NEAR-INFRARED fluorochromes for use in spectral flow cytometry," *Cytometry A* **101**(10), 835–845 (2022).
 76. J. Zhou, Y. Yang, and C. Zhang, "Toward Biocompatible Semiconductor Quantum Dots: From Biosynthesis and Bioconjugation to Biomedical Application," *Chem. Rev.* **115**(21), 11669–11717 (2015).
 77. J. F. Hainfeld and F. R. Furuya, "A 1.4-nm gold cluster covalently attached to antibodies improves immunolabeling," *J. Histochem. Cytochem.* **40**(2), 177–184 (1992).

78. T. Ichikawa, D. Wang, K. Miyazawa, K. Miyata, M. Oshima, and T. Fukuma, "Chemical fixation creates nanoscale clusters on the cell surface by aggregating membrane proteins," *Commun. Biol.* **5**(1), 487 (2022).
79. Y. Li, L. M. Almassalha, J. E. Chandler, X. Zhou, Y. E. Stypula-Cyrus, K. A. Hujsak, E. W. Roth, R. Bleher, H. Subramanian, I. Szleifer, V. P. Dravid, and V. Backman, "The effects of chemical fixation on the cellular nanostructure," *Exp. Cell Res.* **358**(2), 253–259 (2017).
80. A. Idziak, V. V. G. K. Inavalli, S. Bancelin, M. Arizono, and U. V. Nägerl, "The Impact of Chemical Fixation on the Microanatomy of Mouse Organotypic Hippocampal Slices," *eneuro* **10**(9), ENEURO.0104-23.2023 (2023).
81. V. Lučić, F. Förster, and W. Baumeister, "STRUCTURAL STUDIES BY ELECTRON TOMOGRAPHY: From Cells to Molecules," *Annu. Rev. Biochem.* **74**(1), 833–865 (2005).
82. T. H. Giddings, E. T. O'Toole, M. Morphew, D. N. Mastronarde, J. R. McIntosh, and M. Winey, "Using rapid freeze and freeze-substitution for the preparation of yeast cells for electron microscopy and three-dimensional analysis," in *Methods in Cell Biology* (Elsevier, 2001), **67**, pp. 27–42.
83. M. H. Laporte, N. Klena, V. Hamel, and P. Guichard, "Visualizing the native cellular organization by coupling cryofixation with expansion microscopy (Cryo-ExM)," *Nat. Methods* 1–7 (2022).
84. F. Cardarelli, L. Digiacomo, C. Marchini, A. Amici, F. Salomone, G. Fiume, A. Rossetta, E. Gratton, D. Pozzi, and G. Caracciolo, "The intracellular trafficking mechanism of Lipofectamine-based transfection reagents and its implication for gene delivery," *Sci. Rep.* **6**(1), 25879 (2016).
85. S. Hennig, S. van de Linde, M. Lummer, M. Simonis, T. Huser, and M. Sauer, "Instant Live-Cell Super-Resolution Imaging of Cellular Structures by Nano-injection of Fluorescent Probes," *Nano Lett.* **15**(2), 1374–1381 (2015).
86. A. Sharei, J. Zoldan, A. Adamo, W. Y. Sim, N. Cho, E. Jackson, S. Mao, S. Schneider, M.-J. Han, A. Lytton-Jean, P. A. Basto, S. Jhunjhunwala, J. Lee, D. A. Heller, J. W. Kang, G. C. Hartoularos, K.-S. Kim, D. G. Anderson, R. Langer, and K. F. Jensen, "A vector-free microfluidic platform for intracellular delivery," *Proc. Natl. Acad. Sci.* **110**(6), 2082–2087 (2013).
87. C. J. McKinlay, J. R. Vargas, T. R. Blake, J. W. Hardy, M. Kanada, C. H. Contag, P. A. Wender, and R. M. Waymouth, "Charge-altering releasable transporters (CARTs) for the delivery and release of mRNA in living animals," *Proc. Natl. Acad. Sci.* **114**(4), (2017).
88. Y.-C. Wu, T.-H. Wu, D. L. Clemens, B.-Y. Lee, X. Wen, M. A. Horwitz, M. A. Teitell, and P.-Y. Chiou, "Massively parallel delivery of large cargo into mammalian cells with light pulses," *Nat. Methods* (2015).

Chapter 2

11-Channel Fluorescence Microscopy Imaging with a Single Excitation Wavelength Using Polymer Dots with Spectral Line-scan Microscopy

Work represented in this chapter was contributed by Hwanhee C. Kim, Wenhui Xie, Eunjin Jo, Valentino Browning, and Jiangbo Yu. HCK led the project in experimental design, system setup, software development, sample preparation, image acquisition, and image processing. WX co-developed the custom hardware-control script for the SLiM setup. EJ prepared samples and acquired images on SLiM for the 11-channel dataset. VB performed benchmarking analyses to evaluate spectral unmixing algorithms. JY performed Pdot conjugation to antibodies used in the 11-channel panel. Daniel T. Chiu coordinated the sourcing of Pdots used in this work, and Joshua C. Vaughan supervised the work.

2.1 Abstract

Conventional fluorescence microscopy is a powerful tool in biological research and clinical diagnostics for identifying multiple biomarkers within cells and tissues. However, multiplexed imaging is routinely performed with only three or four colors due to broad emission widths and small Stokes shifts, which leads to spectral overlaps and complicate signal separation. Increasing number of detectable targets often requires iterative labeling, sequential imaging, and specialized microscope or fluidics. Here, we demonstrate a single-excitation hyperspectral imaging system

using polymer dots (Pdots) that resolve up to eleven biomarkers concurrently from a single 405 nm illumination. By integrating line-scan spectral imaging with custom hardware synchronization, we simultaneously capture the full emission spectrum and perform pixel-level spectral unmixing in tissue. This approach provides a practical and scalable hyperspectral imaging framework that can be readily adapted to other fluorescence microscopy platforms, enabling high-throughput, high-content multicolor imaging of complex biological system.

2.2 Introduction

In current biomedical research, fluorescence microscopy has become an indispensable tool owing to its capacity to provide spatially resolved detection of biomarkers in a complex biological environment with great sensitivity and specificity [1]. Techniques such as FLARE [2] and Pan-ExM [3] provide global structure context through direct covalent labeling abundant biomolecules (e.g., carbohydrates, proteins, DNA), whereas fluorophore-conjugated antibodies enable targeted detection and quantification of specific proteins [4]. Routinely, these multiplexed fluorescence microscopy techniques achieve three or four channels due to the broad (30-60 nm) emission spectra of organic fluorophores and small Stokes shifts (20-30 nm), which results in substantial spectral overlap [1]. To overcome this number of channels limitation, multiplexing strategies based on sequential labeling and imaging have been developed [5–7]. These approaches rely on iterative cycles of multistep labeling, imaging, and antibody/fluorophore removal (e.g., antibody stripping, fluorophore inactivation), enabling highly multiplexed imaging (>60-plex) from a single biological specimen. However, these workflows are time-intensive, and often require fluidic control and specialized instruments, restricting their scalability and routine use in tissues imaging applications [8,9]. In addition, repeated excitation increases unwanted photobleaching, while

subsequent acquisitions introduce challenges with image registration and signal consistency [10]. Hyperspectral microscopy provides an alternative approach by capturing the full emission spectrum at each pixel by dispersing the emission spectrum with a prism or diffraction grating. Through spectral binning and computational unmixing, hyperspectral techniques have achieved multiplexing ranging from 6-8 channels to > 100-plex combinatorial implementations [9,11,12].

A similar shift has transformed the field of fluorescence-based flow cytometry, where spectral analysis expanded multiplexing by collecting fluorescence across an array of detector channels rather than assigning each marker to individual bandpass filter channel. In this setup, fluorophores with partially overlapping emission spectra can be densely packed within the available spectral space and computationally resolved through spectral unmixing. However, the number of simultaneously detectable channels and the effectiveness of spectral unmixing still depends largely on the spectral properties of fluorophores themselves. While specialized fluorophores, such as long-Stokes-shifts dyes (60-200 nm) and tandem dyes, can expand the accessible spectral space, they often suffer reduced brightness, increased spectral broadening, and variability in performance [13–15]. Quantum dots offer narrow emission spectra and larger Stokes shifts [10,16], making them attractive for multiplexed detection. However, the broad absorption profile spanning the UV to visible range complicates selective excitation. More recently, polymer dots (Pdots) have emerged as a promising probe for multiplexed imaging [17–19]. By engineering their absorption and emission properties, these bright fluorescent semiconducting polymer nanoparticles offer tunable Stokes shifts and high brightness, enabling excitation-multiplexed imaging up to ~21 channels in sequential imaging workflows [18,20].

Here, we present a highly multiplexed approach that adapts the spectral unmixing concept commonly used in flow cytometry to fluorescence microscopy. By combining a spectrally dense panel of 405 nm excitable Pdots with hyperspectral line-scan detection, this strategy packs Pdot emission profiles across visible and near-infrared spectrum and resolve them computationally using pixel-level spectral unmixing. Rather than relying on discrete filter channels, this system acquires spectrally resolved images across large tissue with synchronized hardware control (Fig. 2.1). Using a single round of labeling and imaging, we resolve 11 distinct biomarkers in kidney tissue (50 μm) with minimal signal crosstalk, demonstrating a practical method towards high-throughput multiplexed tissue imaging.

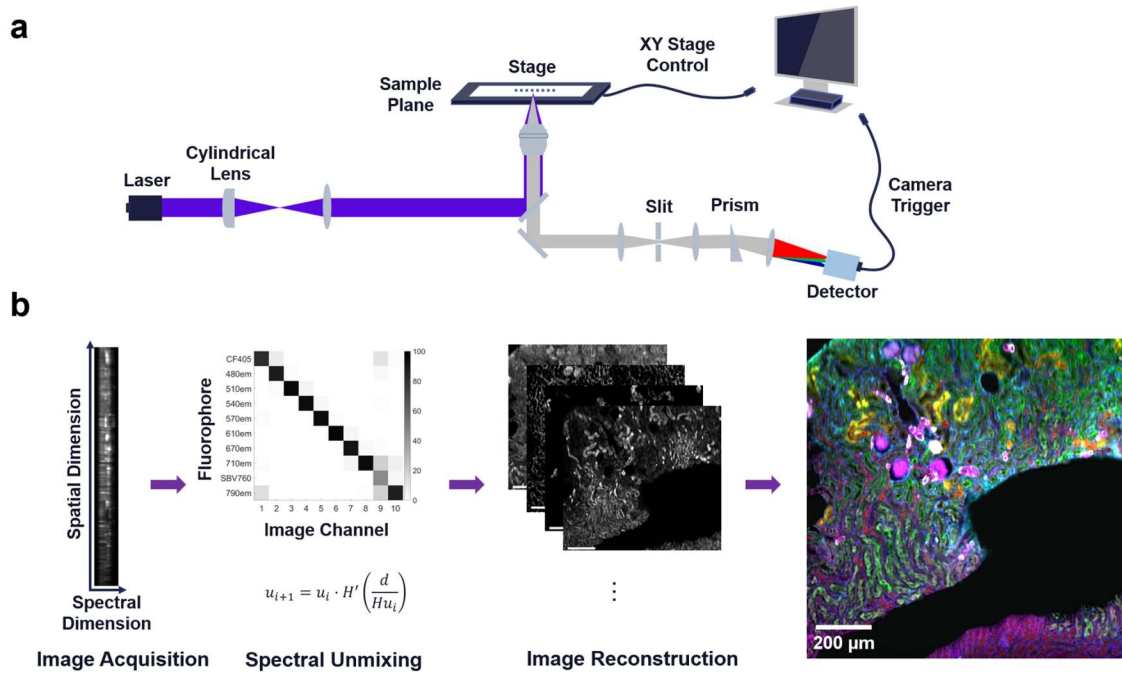


Fig. 2.1 System Overview and Imaging Processing Workflow. (a) Schematic of the line-scan hyperspectral imaging system. A 405 nm laser beam is shaped into a line focus at the sample plane through introduction of a cylindrical lens in the beam path. Sample fluorescence from the illuminated line is collected through the emission path, passed through a long pass filter and an

adjustable slit, dispersed by a prism, and imaged onto the detector. Laser modulation, stage movement, and camera triggering are synchronized through custom control software. (b) Image-processing workflow after spectral acquisition. Each acquired frame contains one spatial dimension and one spectral dimension. Spectral unmixing is performed using the measured calibration matrix, followed by image reconstruction to generate registered multicolor images.

2.3 Methods

2.3.1 System design

In our line-scan hyperspectral system, a collimated 405 nm laser is focused to a line illumination at the sample plane by introducing a cylindrical Lens into the excitation path (Thorlabs; LJ1653RM-A; $f = 200.0$ mm, Ø1", N-BK7 mounted, ARC 350–700). Fluorescence emitted from the specimen was recorded such that one camera axis corresponded to the spatial position along the illuminated line and the perpendicular camera axis corresponded to the dispersed spectral dimension (Fig. 2.1b). Emitted fluorescence is passed through an adjustable mechanical slit (Thorlabs; VA100) set at approximately 280 μm at the intermediate image plane to avoid clipping the in-focus signal while providing partial rejection of out-of-focus fluorescence before spectral dispersion. We spectrally dispersed the fluorescence emission using a wedge prism (Thorlabs; PS812-A; 6° beam deviation, AR coating: 350–700 nm) located at the Fourier plane of a two-lens telescope constructed from a pair of anti-reflection-coated doublet lenses (400–700 nm, $f = 100$ mm; Thorlabs; AC254-100-A-ML). Images were recorded on a scientific camera (Hamamatsu; C13440-20CU).

Custom software was written in Python to coordinate operation of the 405nm laser, camera, and stage). The software utilized a multifunctional data acquisition card (National Instruments; PCI-6229) to initiate laser excitation through direct analog voltage control, to drive continuous motion of the sample stage (ASI; PZ-2500), and to generate camera trigger waveforms at appropriate intervals. This synchronization produced evenly spaced line acquisition across the sample during continuous stage scanning.

2.3.2 Detection parameter and calibration

Camera frames were acquired at approximately 40 Hz using the full 2048×2048 -pixel detector area of the camera. Post-acquisition, images were cropped to a 1000×57 -pixel region, where 1000-pixel axis corresponded to the spatial dimension and the 57-pixel axis corresponded to the dispersed spectral dimension (Fig. 2.1b). In this configuration, the 57-pixel spectral axis covered an emission range of approximately 440-800 nm, corresponding to an average dispersion of 6.7 nm/pixel. For tissue imaging, each scan covered a 4000 μm range in the scan direction. Large tissue regions were acquired by tiling adjacent scans with a 700 μm range. With a 4 $\times$ Nikon CFI60 Plan Apochromat Lambda D (Nikon; MRD70040), the line spacing was set to 1.62 μm .

Calibration spectra were measured from a ~ 2 μL of a diluted solution of Pdot-antibody conjugates in a flow channel. The flow channel was constructed by sandwiching two pieces of double-sided tape (~ 100 μm thickness) between a #1.5 cover glass and a microscope slide to create a narrow channel into which the Pdot solution was pipetted.

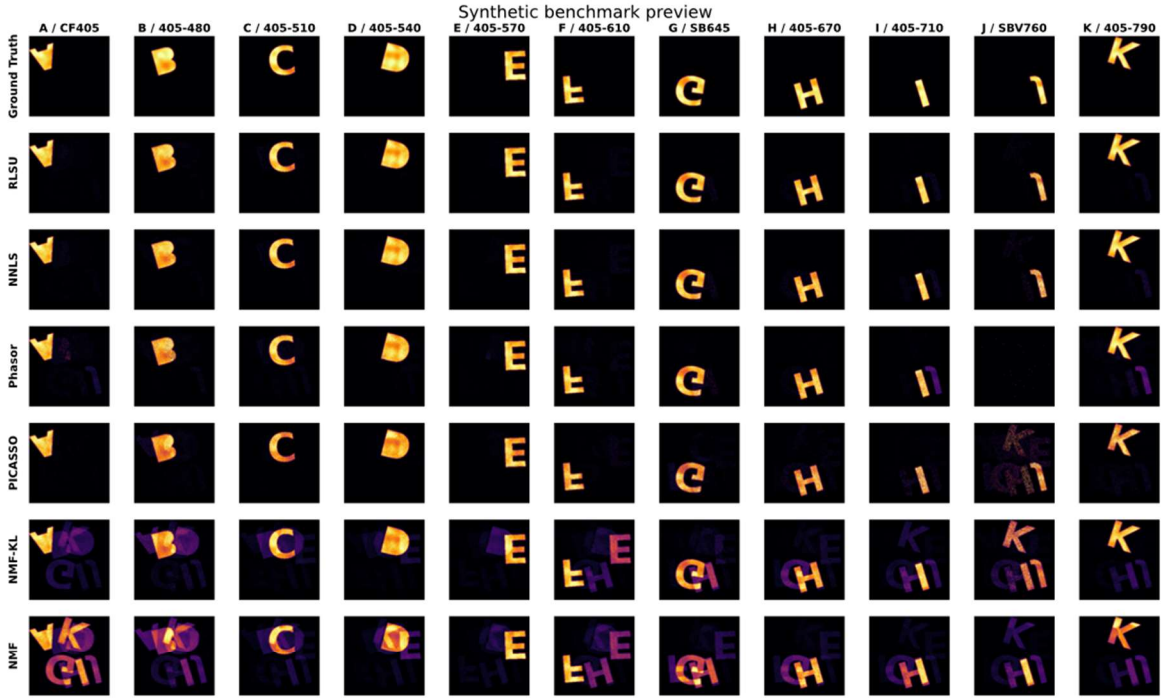
2.3.3 Spectral unmixing

Pixel-by-pixel spectral unmixing was required to separate the emissions from densely overlapping Pdot spectra. To select an appropriate unmixing approach, we benchmarked several spectral unmixing algorithms using synthetic image generated from calibration spectra and known ground-truth abundance with added Poisson noise (Fig. 2.2a). The tested methods include non-negative least-squares (NNLS), phasor-based unmixing [21], Nonnegative Matrix Factorization (NMF) [22], Nonnegative Matrix Factorization-Kullback-Leibler Divergence (NMF-KL) [23], and PICASSO (Process of ultra-multiplexed Imaging of biomoleCules viA the unmixing of the Signals of Spectrally Overlapping fluorophores) [24], and Richardson-Lucy spectral unmixing algorithm (RLSU) [25]. Among these algorithms, RLSU showed strong quantitative performance in synthetic comparisons, producing correlative unmixed images to ground-truth image. The mean root-mean square error (RMSE) value was calculated across all channels, and per-channel RMSE was used to assess wavelength-dependent differences in unmixing performance (Fig. 2.2b). To further evaluate unmixing specificity, the spectral crosstalk was quantified by applying unmixing workflow to individual calibration spectra to measure the fraction of recovered signals assigned to non-corresponding Pdot channels.

RLSU had previously been adapted from iterative Richardson-Lucy deconvolution for uses in spectral unmixing. In this implementation, the measured spectrum at each pixel was treated as a combination of non-negative abundance of each reference spectra from the calibration panel. Starting from an initial estimate, RLSU iteratively updates the channel weights (i.e., abundance) using a multiplicative correction term based on the ratio between the measured spectrum and

predicted spectrum. Iterations were continued until the relative change between channel weights reached a stopping point (e.g., $1\text{E-}6$) or the maximum iteration number.

a



b

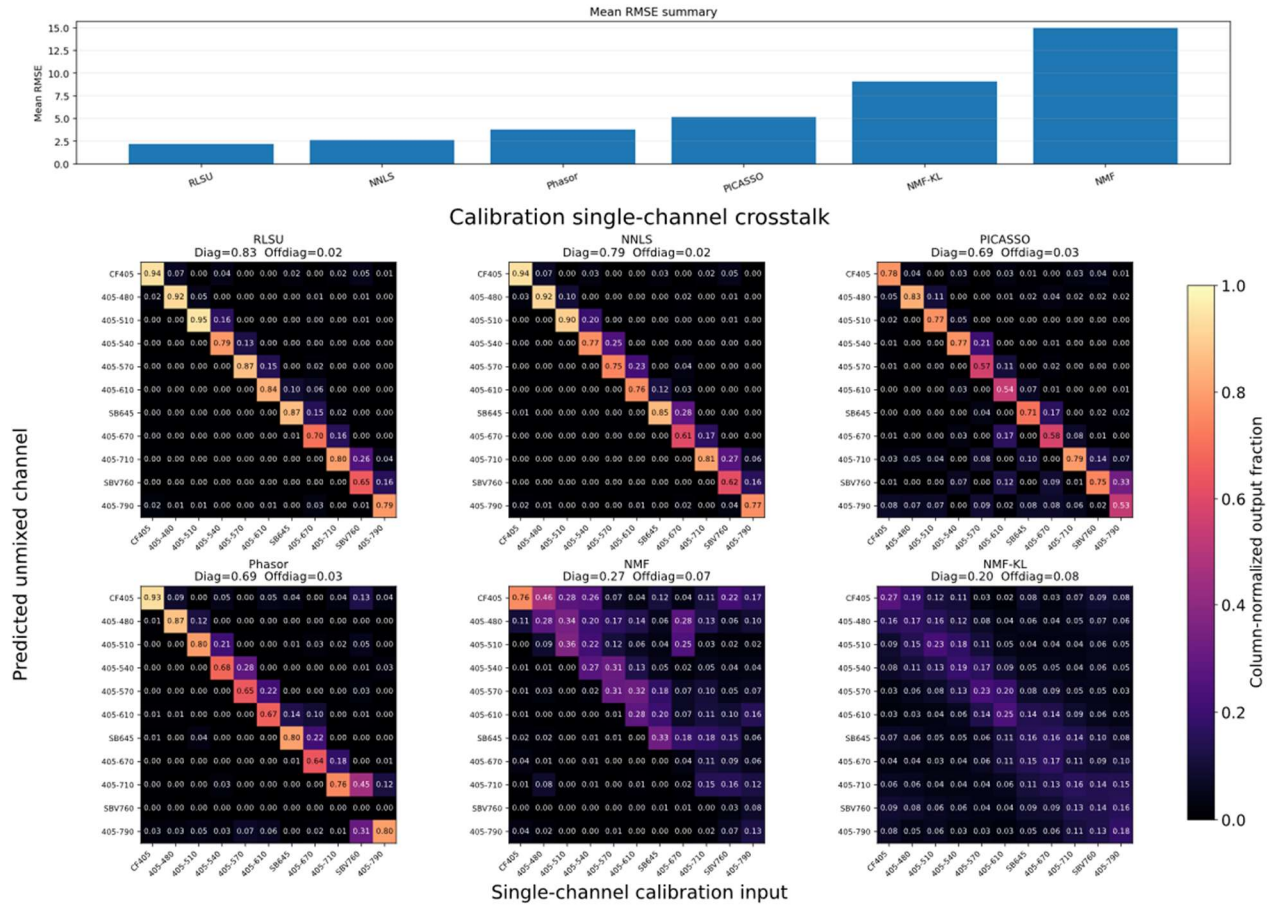


Fig. 2.2 Comparisons of spectral unmixing algorithm performance. (a) Representative synthetic 11-channel images showing the ground-truth input with added Poisson noise and unmixed outputs for NNLS, RLSU, phasor-based, PICASSO, NMF, and NMF-KL unmixing. Each column represents one Pdot channel, and residual signal in non-corresponding channels reflect channel leakage. (b) Quantitative comparison of unmixing performance. The top plot summarizes the average RMSE for RLSU, NNLS, PICASSO, phasor analysis, NMF, and NMF-KL, where lower RMSE indicate closer correspondence in abundance to ground-truth. Lower matrices represents single-channel crosstalk analysis comparing unmixed outputs against calibration spectra as individual inputs. Each matrix reports the fraction of predicted signals assigned to each unmixed channel after unmixing one Pdot spectrum at a time. Strong diagonal signal indicates correct channel predictions, while off-diagonal signal indicates spectral crosstalk between channels. RLSU = Richardson-Lucy spectral unmixing; NNLS = Non-negative least-square; PICASSO = Process of ultra-multiplexed Imaging of biomolecules via the unmixing of the Signals of Spectrally Overlapping fluorophores; NMF = Nonnegative Matrix Factorization; NMF-KL = Nonnegative Matrix Factorization-Kullback-Leibler Divergence

2.3.4 Sample preparation

Primary antibody and lectin probes were conjugated to Pdots as previously reported using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling [18]. The probe panel included rabbit anti-LRP2 (Invitrogen; PA5-92032), goat anti-podxl (R&D Systems; AF1556), rabbit anti-parvalbumin (Novus Biologicals; NB120-11427), rabbit anti-collagen IV (Abcam; ab6586), goat anti-podoplanin (R&D Systems; AF3244), mouse IgG2a anti- α -SMA (Millipore Sigma; A5228-

200UL), rat anti-mouse CD31 StarBright Violet 760 (Bio-Rad Laboratories; MCA2388SBV760), wheat germ agglutinin (Vector Labs; L-1020-25), and LEL (Vector Labs; L-1170-2).

6-month-old mouse kidney was perfused with 4% paraformaldehyde, harvested, and sectioned at 50 μm thickness using a vibratome. Tissue sections were stored in $1\times$ phosphate-buffered saline (PBS) with azide until immunostaining. Individual sections were transferred to μ -Slide 8-well chambers (Ibidi; 80826) and incubated in blocking/permeabilization buffer containing 4% bovine serum albumin (BSA) and 0.01% Triton X-100 for 1 hour at room temperature. Antibody and lectin probes were diluted in blocking buffer at final concentrations of 0.25–50 $\mu\text{g/mL}$ and incubated with tissue sections overnight at 4 $^{\circ}\text{C}$. After three 10-minute washes with blocking buffer at room temperature, samples were stained with phalloidin CruzFluor™ 405 conjugate (Santa Cruz Biotechnology; sc-363790) at $1\times$ working concentration for 10 minutes at room temperature. Samples were then washed three times with PBS-azide for 10 minutes at room temperature, mounted onto microscope slides (Fisher Scientific; 12-550D), and covered with rectangular cover glasses (Fisher Scientific; 12-541-033) before imaging.

2.4 Results

We first identified a fluorophore panel excitable with 405nm light but with emission peaks separated by at least 15 nm under 405 nm excitation. The panel included compatible Pdot probes and one small-molecule dye, with emission spectra either measured in-house using a fluorescence spectrophotometer (Edinburgh Instruments; FS5 Spectrofluorometer) or obtained from manufacturer-provided datasheets (Fig. 2.3a). We then measured each fluorophore individually using the line-scan hyperspectral system to generate system-specific emission profiles for the 11-

channel panel. These measurements confirmed that each fluorophore produced sufficient signal and spectral separation for computational unmixing. Because the prism dispersion is non-linear, the effective wavelength spacing varied across the detector. In addition, it is likely that the wavelength-dependent transmission efficiency of the microscope emission path and detection efficiency of the camera impart some distortions of the spectral shape. All things considered, in the red and far-red regions, emissions centered at 710, 760, and 790 nm were separated by approximately 2–3 pixels, corresponding to ~13–20 nm in wavelength separation. This separation is comparable to the practical spectral resolution reported for many fluorescence hyperspectral imaging systems [25,26].

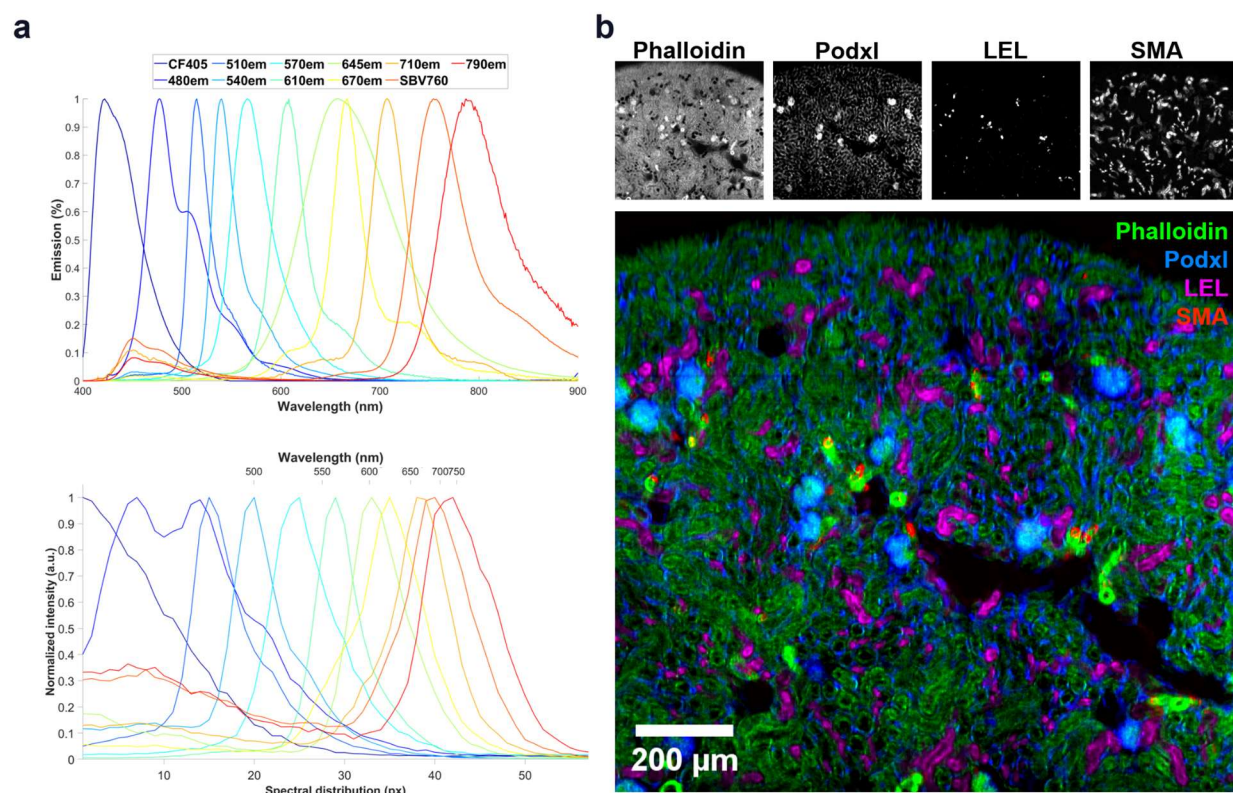


Fig. 2.3 11-channel dye panel characterization and four-channel tissue unmixing. (a) The top plot shows reference spectra from manufacturer-provided data for commercially available probes

and fluorometer measurements for Pdot probes. Bottom plot shows the corresponding spectral measured through hyperspectral microscope. The bottom x-axis shows the spectral distribution in the pixel axis, and the top x-axis shows the wavelength calibration estimated from measured peak positions. (b) Four-channel validation of tissue imaging and spectral unmixing in mouse kidney. The panel shows individual unmixed channel and the merged image for phalloidin: CruzFluor 405, Podxl: 510em, LEL: 670em, and SMA: 790em.

In an initial test of our system, we sought to resolve a four-channel imaging panel with 405 nm excitation. We stained mouse kidney tissue with four probes whose emission maxima centered at 440, 510, 610, and 670 nm. These wavelengths were selected to approximate the fluorescence emission of small-molecule fluorophores commonly used with standard 405, 488, 561, and 647 nm excitation on commercial epifluorescence or confocal microscopes. After RLSU unmixing, the four-channel data showed spatially distinct physiological features corresponding with four probes with minimal overlap between channels (Fig. 2.3b). Phalloidin labeled actin-rich structures, LEL labeled *N*-acetylglucosamine (GlcNAc) oligomers, podocalyxin (podxl) labeled podocytes, and SMA labeled vascular smooth muscle cells in kidney tissue. This experiment served as an initial validation to demonstrate the feasibility of multiplexing with Pdots using hyperspectral system, before extending the application to full 11-channels.

To further evaluate the unmixing specificity of RLSU, we stained mouse kidney tissue with a 4-fluorophore subset selected from the full 11-channel panel and then unmixed using the full 11-channel calibration matrix (Fig. 2.4). The signals were predominantly assigned to the four expected fluorophore channels, with low levels of signal evident mainly in the spectrally adjacent

absent channels. The individual unmixed images showed staining patterns consistent with the expected biomarker distribution, supporting the specificity of RLSU when samples contain only a subset of the full calibration panel.

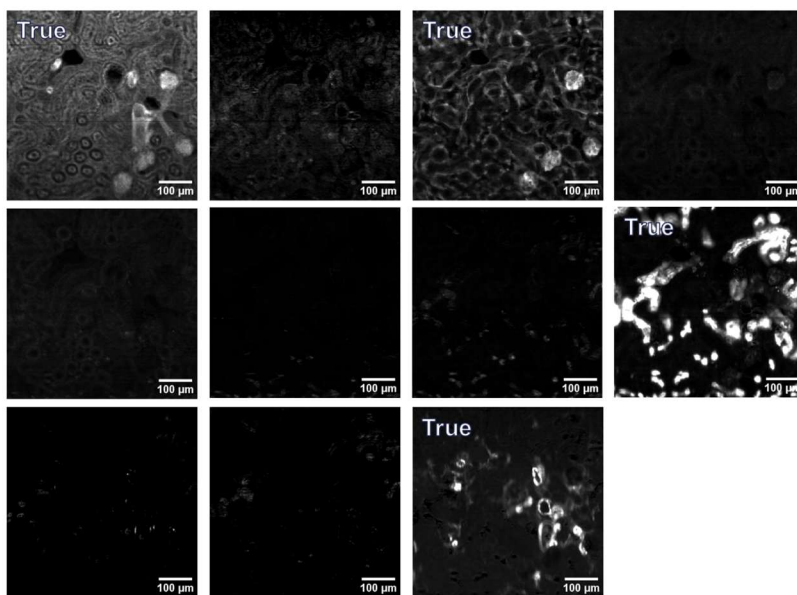


Fig. 2.4 Subset labeling and unmixing. Mouse kidney tissue was stained with a 4-fluorophore subset panel comprising phalloidin (CF405), podxl (510em), LEL (670em), α -SMA (790em). These probes were selected to span a broad portion of the spectral range and to label spatially distinct tissue features. The sample was unmixed using the full 11-channel calibration matrix. Panels labeled “True” indicate the expected fluorophores present in the sample, while the remaining panels correspond to absent channels. Signals were recovered primarily in the expected channels with minimal crosstalk into absent channels.

To demonstrate the central capability of our system for single-excitation multiplexed imaging, we stained mouse kidney tissue with an 11-channel probe panel and imaged using a single 405 nm excitation source (Fig. 2.5). After RLSU unmixing, individual channels showed distinct

staining patterns consistent with the expected distribution for each probe, and the merged image preserved clear channel-specific features. Using programmable scanning and tiling parameters, we further acquired a larger composite image spanning the entire tissue section beyond a single field of view (Fig. 2.6).

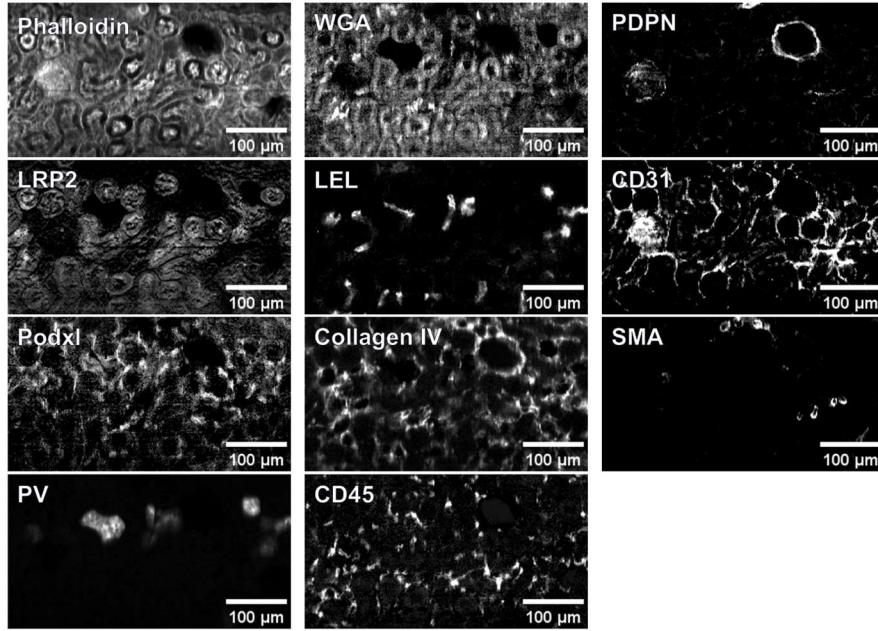


Fig. 2.5 11-channel mouse kidney tissue imaging and spectral unmixing. *Multiplexed imaging of 50 µm-thick mouse kidney tissue using 11-channel probe panel and single 405 nm excitation. Each grayscale image shows individual unmixed channel after RLSU spectral unmixing. Labels indicate the targeted structure or biomarker and the corresponding fluorophore channel: phalloidin (CF405), LRP2 = megalin/low density lipoprotein-related protein 2 (480em), podxl = podocalyxin (510em), PV = parvalbumin (540em), WGA = wheat germ agglutinin (570em), Collagen IV (610em), CD45 (SB645), LEL = lycopersicon esculentum lectin (670em), PDPN = podoplanin (710em), CD31 (SBV760), SMA = alpha-smooth muscle actin (790em), .*

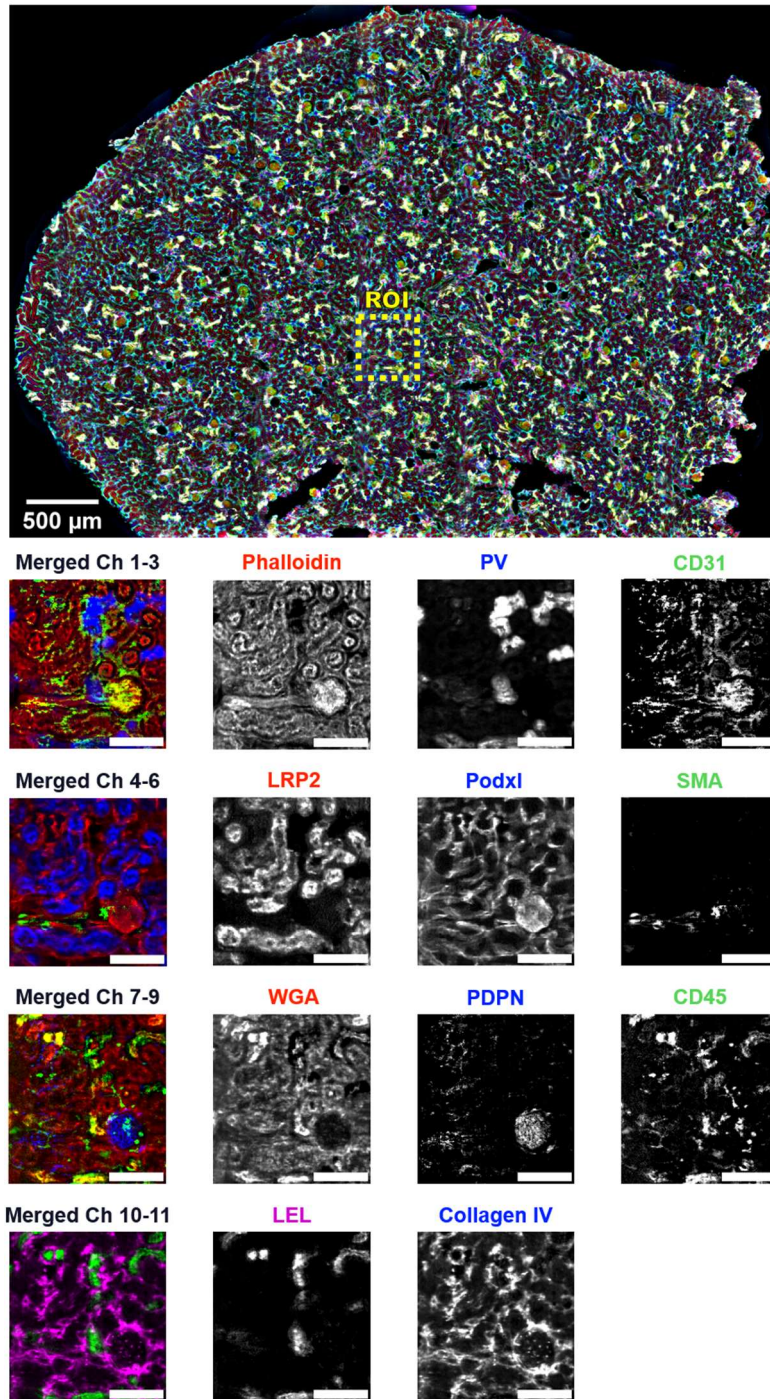


Fig. 2.6 Large area 11-channel multiplexed imaging of mouse kidney tissue. Mouse kidney tissue was labeled with 11-probe panel and was imaged using hyperspectral line-scan tiling, and reconstructed after image stitching and spectral unmixing. The full-tissue image display representative 7-color merge showing phalloidin, Podxl, PV, WGA, collagen IV, LEL, and α -SMA

to visualize the tissue environment across larger area extending beyond a single field of view. The yellow dashed line indicate the ROI expanded below. Zoomed ROI panels show grouped merged and individual unmixed images of 11-biomarker panel, demonstrating the capability to recover biomarker-specific tissue structures from the large-region dataset. Scale bar for zoomed ROI, 100 μm .

Eleven-channel multiplexed imaging of a mouse kidney tissue section acquired by tiling and reconstructed after image stitching. The merged image represent seven of eleven channels, showing phalloidin, Podxl, PV, WGA, collagen IV, LEL, and α -SMA channels across a tissue region extending a single field of view.

2.5 Conclusions and discussion

In this work, we demonstrated that a line-scan hyperspectral imaging system can concurrently detect and separate eleven spectrally distinct biomarkers using a single 405 nm excitation source. By integrating line-scan spectral detection with synchronized hardware control, the system enables automated acquisition across large tissue regions. Using an adapted RLSU algorithm, we unmixed fluorophores with closely spaced emission maxima and recovered distinct biomarker staining patterns with minimal crosstalk. We also verified the system's compatibility with a commercial small-molecule probe, supporting broader applicability beyond Pdot-based labeling.

A major future direction is extending this method to slit-confocal volumetric multiplexed imaging. Because the system already includes an adjustable detection slit, the optical design can

be adapted towards slit-confocal sectioning by optimizing the line illumination width, slit aperture, and axial scanning. This would allow Pdot signals to be acquired across tissue depth while reducing out-of-focus background, which is critical for imaging 3D tissue environments. A second direction is to add excitation multiplexing using 488, 561, and 647 nm wavelengths. Implementing additional excitation imaging rounds could enable integration of other excitation-multiplexed polymer dot series [20,27] and compatible small-molecule dyes, potentially increasing the number of detectable biomarkers in a single round of labeling and imaging to more than 30 channels.

Although RLSU showed strong unmixing performance in analysis of our synthetic data, different algorithms exhibited varying performance across individual channels. This suggests that future implementations of spectral unmixing algorithms could benefit from more flexible strategies that consider both accuracy and computational speed. For example, faster methods such as NNLS and phasor-based unmixing could provide initial estimates and images for review and quality control, while more rigorous methods such as RLSU could be prioritized for unmixing fluorophores with greater spectral overlap. An ensemble approach, where algorithm selection or weighting is informed by per-channel RMSE, may improve channel-specific unmixing performance without uniformly applying the most computationally intensive algorithms across all channels.

Beyond image reconstruction, this multiplexed hyperspectral imaging approach may be useful for spatial cytometry in tissue, where cell-surface biomarkers, including cluster of differentiation (CD) proteins, could be profiled while preserving their spatial organization within the tissue microenvironment. Similar capabilities have been demonstrated in other imaging

cytometry techniques, such as CODEX and mass cytometry [6,28]. For broader use in tissue cytometry, however, autofluorescence remains an additional challenge due to high levels of endogenous tissue fluorescence that can obscure or distort fluorophore spectra during unmixing. Future work could incorporate autofluorescence reference spectra empirically or by combining spectral signatures from known tissue components, such as NADH/NADPH and lipids [29]. Separating this background component could improve spectral unmixing while also retaining autofluorescence-derived structural context.

2.6 References

1. J. W. Lichtman and J.-A. Conchello, "Fluorescence microscopy," *Nat. Methods* **2**(12), 910–919 (2005).
2. M. Y. Lee, C. Mao, A. K. Glaser, M. A. Woodworth, A. R. Halpern, A. Ali, J. T. C. Liu, and J. C. Vaughan, "Fluorescent labeling of abundant reactive entities (FLARE) for cleared-tissue and super-resolution microscopy," *Nat. Protoc.* **17**(3), 819–846 (2022).
3. O. M'Saad and J. Bewersdorf, "Light microscopy of proteins in their ultrastructural context," *Nat. Commun.* **11**(1), 3850 (2020).
4. C. R. Taylor and R. M. Levenson, "Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II," *Histopathology* **49**(4), 411–424 (2006).
5. J.-R. Lin, M. Fallahi-Sichani, and P. K. Sorger, "Highly multiplexed imaging of single cells using a high-throughput cyclic immunofluorescence method," *Nat. Commun.* **6**, 8390 (2015).
6. Y. Goltsev, N. Samusik, J. Kennedy-Darling, S. Bhate, M. Hale, G. Vazquez, S. Black, and G. P. Nolan, "Deep Profiling of Mouse Splenic Architecture with CODEX Multiplexed Imaging," *Cell* **174**(4), 968-981.e15 (2018).
7. S. K. Saka, Y. Wang, J. Y. Kishi, A. Zhu, Y. Zeng, W. Xie, K. Kirli, C. Yapp, M. Cicconet, B. J. Beliveau, S. W. Lapan, S. Yin, M. Lin, E. S. Boyden, P. S. Kaeser, G. Pihan, G. M. Church, and P. Yin, "Immuno-SABER enables highly multiplexed and amplified protein imaging in tissues," *Nat. Biotechnol.* 1–11 (2019).
8. J. W. Hickey, E. K. Neumann, A. J. Radtke, J. M. Camarillo, R. T. Beuschel, A. Albanese, E. McDonough, J. Hatler, A. E. Wiblin, J. Fisher, J. Croteau, E. C. Small, A. Sood, R. M. Caprioli, R. M. Angelo, G. P. Nolan, K. Chung, S. M. Hewitt, R. N. Germain, J. M.

Spraggins, E. Lundberg, M. P. Snyder, N. L. Kelleher, and S. K. Saka, "Spatial mapping of protein composition and tissue organization: a primer for multiplexed antibody-based imaging," *Nat. Methods* (2021).

9. A. M. Valm, R. Oldenbourg, and G. G. Borisy, "Multiplexed Spectral Imaging of 120 Different Fluorescent Labels," *PLOS ONE* **11**(7), e0158495 (2016).

10. M. Bruchez, M. Moronne, P. Gin, S. Weiss, and A. P. Alivisatos, "Semiconductor Nanocrystals as Fluorescent Biological Labels," *Science* **281**(5385), 2013–2016 (1998).

11. K. Chen, R. Yan, L. Xiang, and K. Xu, "Excitation spectral microscopy for highly multiplexed fluorescence imaging and quantitative biosensing," *Light Sci. Appl.* **10**(1), 97 (2021).

12. A. Kumar, K. E. McNally, Y. Zhang, A. Haslett-Saunders, X. Wang, J. Guillem-Marti, D. Lee, B. Huang, R. R. Kay, D. Baker, E. Derivery, and J. D. Manton, "Multispectral live-cell imaging with uncompromised spatiotemporal resolution," *bioRxiv* (2024).

13. M. H. W. Stopel, C. Blum, and V. Subramaniam, "Excitation Spectra and Stokes Shift Measurements of Single Organic Dyes at Room Temperature," *J. Phys. Chem. Lett.* **5**(18), 3259–3264 (2014).

14. P. Horváth, P. Šebej, T. Šolomek, and P. Klán, "Small-Molecule Fluorophores with Large Stokes Shifts: 9-Iminopyronin Analogues as Clickable Tags," *J. Org. Chem.* **80**(3), 1299–1311 (2015).

15. I. Likhokin, R. Lincoln, M. L. Bossi, A. N. Butkevich, and S. W. Hell, "Photoactivatable Large Stokes Shift Fluorophores for Multicolor Nanoscopy," *J. Am. Chem. Soc.* **145**(3), 1530–1534 (2023).

16. I. L. Medintz, H. T. Uyeda, E. R. Goldman, and H. Mattoussi, "Quantum dot bioconjugates for imaging, labelling and sensing," *Nat. Mater.* **4**(6), 435–446 (2005).

17. C. Wu and D. T. Chiu, "Highly Fluorescent Semiconducting Polymer Dots for Biology and Medicine," *Angew. Chem. Int. Ed.* **52**(11), 3086–3109 (2013).

18. J. Yu, Y. Rong, C.-T. Kuo, X.-H. Zhou, and D. T. Chiu, "Recent Advances in the Development of Highly Luminescent Semiconducting Polymer Dots and Nanoparticles for Biological Imaging and Medicine," *Anal. Chem.* **89**(1), 42–56 (2017).

19. C. Wu, T. Schneider, M. Zeigler, J. Yu, P. G. Schiro, D. R. Burnham, J. D. McNeill, and D. T. Chiu, "Bioconjugation of Ultrabright Semiconducting Polymer Dots for Specific Cellular Targeting," *J. Am. Chem. Soc.* **132**(43), 15410–15417 (2010).

20. Z. Guo, C. Poudel, M. C. Sarfatis, J. Yu, M. Wong, D. T. Chiu, and J. C. Vaughan, "Highly multiplexed fluorescence microscopy with spectrally tunable semiconducting polymer dots," *ScienceAdvances* **10**, eadk8829 (2024).

21. F. Fereidouni, A. N. Bader, and H. C. Gerritsen, "Spectral phasor analysis allows rapid and reliable unmixing of fluorescence microscopy spectral images," *Opt. Express* **20**(12), 12729 (2012).

22. R. A. Neher, M. Mitkovski, F. Kirchhoff, E. Neher, F. J. Theis, and A. Zeug, "Blind Source Separation Techniques for the Decomposition of Multiply Labeled Fluorescence Images," *Biophys. J.* **96**(9), 3791–3800 (2009).
23. J. P. Nkurunziza, F. Nahayo, and N. Gillis, "Orthogonal Nonnegative Matrix Factorization with the Kullback-Leibler divergence," *Pattern Recognit. Lett.* **197**, 353–358 (2025).
24. J. Seo, Y. Sim, J. Kim, H. Kim, I. Cho, H. Nam, Y.-G. Yoon, and J.-B. Chang, "PICASSO allows ultra-multiplexed fluorescence imaging of spatially overlapping proteins without reference spectra measurements," *Nat. Commun.* **13**(1), 2475 (2022).
25. A. Kumar, K. E. McNally, Y. Zhang, A. Haslett-Saunders, X. Wang, J. Guillem-Marti, D. Lee, B. Huang, S. Stallinga, R. R. Kay, D. Baker, E. Derivery, and J. D. Manton, "Multispectral live-cell imaging with uncompromised spatiotemporal resolution," *Nat. Photonics* **19**, 1146–1156 (2025).
26. R. Wang, Y. Feng, and A. M. Valm, "A framework of multi-view machine learning for biological spectral unmixing of fluorophores with overlapping excitation and emission spectra," *Brief. Bioinform.* **26**(1), bbaf005 (2025).
27. Y. Rong, C. Wu, J. Yu, X. Zhang, F. Ye, M. Zeigler, M. E. Gallina, I.-C. Wu, Y. Zhang, Y.-H. Chan, W. Sun, K. Uvdal, and D. T. Chiu, "Multicolor Fluorescent Semiconducting Polymer Dots with Narrow Emissions and High Brightness," *ACS Nano* **7**(1), 376–384 (2013).
28. C. Giesen, H. A. O. Wang, D. Schapiro, N. Zivanovic, A. Jacobs, B. Hattendorf, P. J. Schüffler, D. Grolimund, J. M. Buhmann, S. Brandt, Z. Varga, P. J. Wild, D. Günther, and B. Bodenmiller, "Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry," *Nat. Methods* **11**(4), 417–422 (2014).
29. B. J. Rossetti, S. A. Wilbert, J. L. Mark Welch, G. G. Borisy, and J. G. Nagy, "Semi-blind sparse affine spectral unmixing of autofluorescence-contaminated micrographs," *Bioinformatics* **36**(3), 910–917 (2020).

Chapter 3

Live-cell Delivery of Nanogold-conjugated Exogenous Probes for Cryo-EM Labeling and Detection

Work represented in this chapter was contributed by Hwanhee C. Kim, Yuka Kurasaka, Ziyu Guo, Joel Quispe, and Wentao Jiang. HCK led the project in workflow optimization, sample preparation, and EM imaging coordination. YK prepared samples and coordinated imaging with JQ and WJ. ZG contributed to the optimization of saponin-induced live-cell delivery of antibody probes. JQ and WJ cryofixed samples and performed electron microscopy imaging. Justin M. Kollman provided access to the electron microscope, and Joshua C. Vaughan supervised the work. This work was supported by funding from the W. M. Keck Foundation.

3.1 Abstract

Investigating nanoscale organization in biological models often requires specialized instruments that are both highly sensitive and specific, whether the goal is to probe chromatin composition using sequencing or to reveal the spatial context of biological structures through microscopy. Electron microscopy, in particular, can offer near-atomic-level resolution compared to optical methods through electron scattering and absorption. However, electron microscopy is often limited in its ability to label specific structures beyond general macromolecular contrast provided by heavy-metal labeling strategies. Labeling becomes even more challenging for cryogenic electron microscopy (cryo-EM), which offers superior sample preservation through

cryofixation but restricts access for exogenous metal-rich probes without extensive procedures such as freeze substitution. To address this shortcoming, I devised a workflow for live-cell delivery of exogenous labels through transient cell membrane permeabilization using saponin to introduce doubly labeled antibody probes prior to cryofixation. I verified the presence of both fluorophore and metal labels through fluorescence microscopy and silver-enhancement-assisted light microscopy. I also confirmed that the cell membrane recovers after a short recovery period in growth medium while the exogenous label remains intracellularly bound to the target structure. With future validation of nanogold labeling in electron microscopy, this chapter presents an ongoing effort to extend multiplexing strategies to cryo-EM applications for specific detection of target proteins within low-contrast environments.

3.2 Introduction

The chemistry driving biological change exists at the interface of molecular interactions, whether in chromatin reorganization, signaling cascades, or cytoskeletal branching. Although biochemical techniques such as small-molecule pull-down assays and sequencing-based methods such as ChIP-seq [1], Hi-C [2], or DNA O-MAP [3] can investigate molecular proximity, these approaches are limited to inferred adjacency through chemical linkage or population-averaged contact frequency and do not explicitly define the spatial localization of components relative to one another. Microscopy remains one of the most effective approaches for probing the spatial architecture of biological systems; however, the resolution of traditional light microscopy is limited by diffraction, making it difficult to resolve structures smaller than approximately 250 nm. While technical advances have enabled super-resolution microscopy to achieve nanoscale resolution, electron microscopy often provides a complementary, higher-resolution view of

biological ultrastructure by directly converting electron scattering and absorption into image contrast.

However, there are costs associated with this gain in resolution, particularly in sample contrast. Despite its superior resolution, electron microscopy of biological samples often has limited intrinsic contrast, making it difficult to distinguish specific macromolecules from the surrounding ultrastructure. Heavy-metal reagents such as osmium tetroxide, uranyl acetate, and lead citrate are commonly used to enhance contrast by increasing electron density within biological samples [4]. Specific labeling strategies, including immunogold labeling [5] a genetically encoded or molecular shape-tags [6], have also been developed to improve molecular identification in electron microscopy. Although powerful, these approaches can be difficult to implement in cryogenic workflows. Cryogenic electron microscopy (cryo-EM), for example, can preserve biological structures in a near-native vitrified state and avoids many perturbative artifacts associated with dehydration, embedding, or chemical fixation used in many conventional EM workflows [7]. However, vitrification restricts further sample manipulation, making it challenging to deliver exogenous labels without extensive additional processing in an already low-throughput pipeline.

To devise an accessible labeling method that can be incorporated into current cryo-EM workflows without substantially increasing processing time, I investigated live-cell delivery of exogenous labels using the transient membrane-permeabilizing properties of saponin, a mild detergent. Saponin-based reversible permeabilization has been used to extract intracellular proteins or introduce large particles such as quantum dots into cells [8,9]. The transient nature of mild

detergents such as saponin allows the cell membrane to recover, preserving the cell's ability to perform cellular processes such as proliferation (Fig. 3.2). In the rest of this chapter, I describe the optimized workflow for cell culture on EM grids, live-cell delivery of antibody probes, cryofixation, and imaging. The goal of this workflow is to introduce specific, electron-dense labels before cryofixation, so that the target macromolecules are localized in preserved cellular ultrastructure. This approach may help reveal spatial organization of targets in a low-contrast cryo-EM environment while minimizing additional processing after vitrification.

3.3 Results

3.3.1 Saponin-induced live-cell delivery of cytoskeleton and organelle targeting antibody probes

To prepare samples for electron microscopy imaging, cells must be cultured on grids coated with appropriate materials that reduce background and provide suitable regions for cell adhesion and imaging. Unlike other live-cell delivery methods that rely on biological mechanisms such as endocytosis or require dedicated instruments or specialized sample-preparation formats [10–14], mild detergents such as diluted Triton X-100 or saponin can be adapted to a wide range of sample configurations. Based on prior evidence that saponin creates holes in the membrane large enough for Qdot-sized macromolecules (~10–15 nm) [9], I optimized a saponin concentration of 0.1 mg/mL to permeabilize the cell membrane sufficiently for intracellular antibody delivery while limiting cell death (Fig. 3.1). Under these conditions, high concentrations of primary anti-tubulin antibody and fluorophore-conjugated secondary antibody (350 nM) were successfully introduced into live cells and labeled intracellular microtubule structures. Similar delivery results were

observed for TOM20 and lamin A/C antibodies, which produced labeling patterns consistent with mitochondria and the nuclear envelope, respectively.

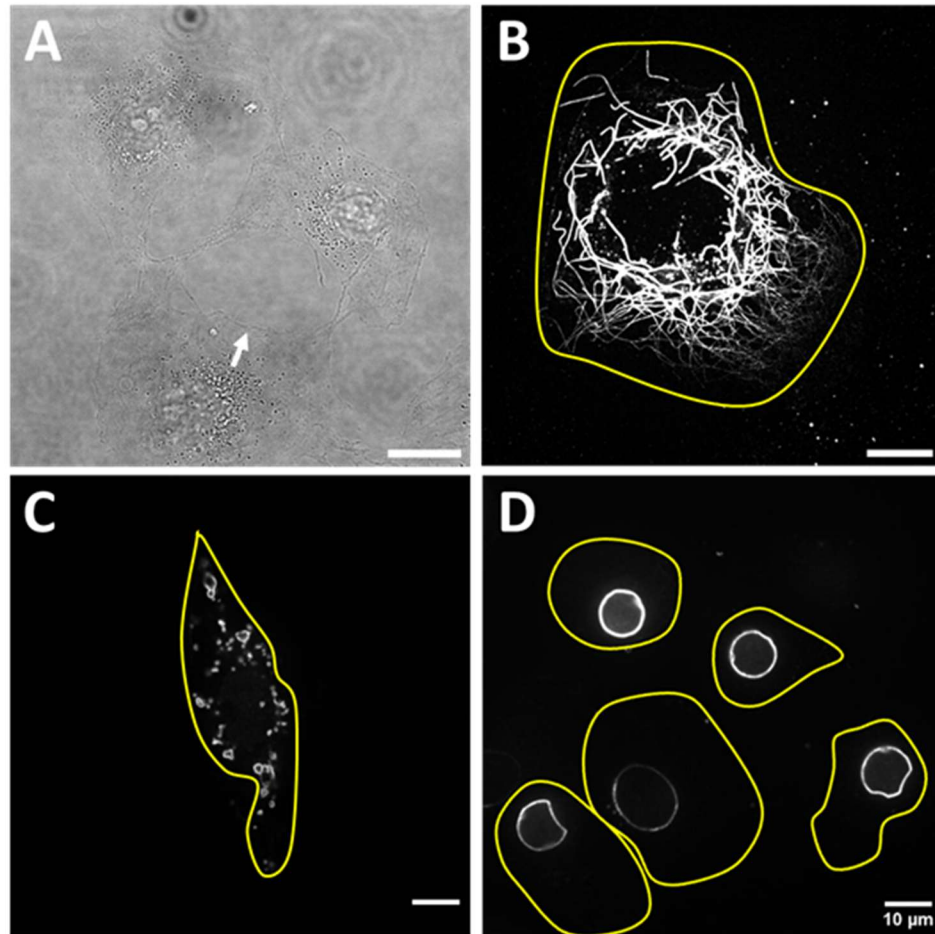


Fig. 3.1 Cells Treated with Saponin followed by Fluorophore-conjugated Antibody Probe Delivery. (a) Cells treated with 0.1 mg/mL saponin show healthy general morphology. (b) Fluorescent probes against microtubules were delivered to cells treated with saponin. Scale bars = 20 μ m. (c,d) Probes against TOM20 and nuclear lamin A were delivered to cells treated with saponin to show mitochondria and nuclear lamina structures respectively. Scale bars = 10 μ m. Samples were fixed after live-cell delivery protocol. Fixed samples were imaged at 60x magnification using brightfield microscopy or spinning disk confocal fluorescence microscopy.

3.3.2 Verifying cell membrane recovery

To determine whether saponin-induced membrane permeability was reversible, I used propidium iodide, a cell-impermeable DNA-targeting fluorescent small molecule, to assess membrane integrity. In saponin-treated cells, propidium iodide produced intracellular fluorescence, confirming membrane permeabilization. After a recovery sequence consisting of 30 minutes of incubation in growth medium at 37 °C, propidium iodide was no longer detected inside the cells, indicating recovery of membrane integrity (Fig. 3.2a). Antibody probes delivered during saponin treatment remained intracellular after recovery and continued to label their target structures (Fig. 3.3c).

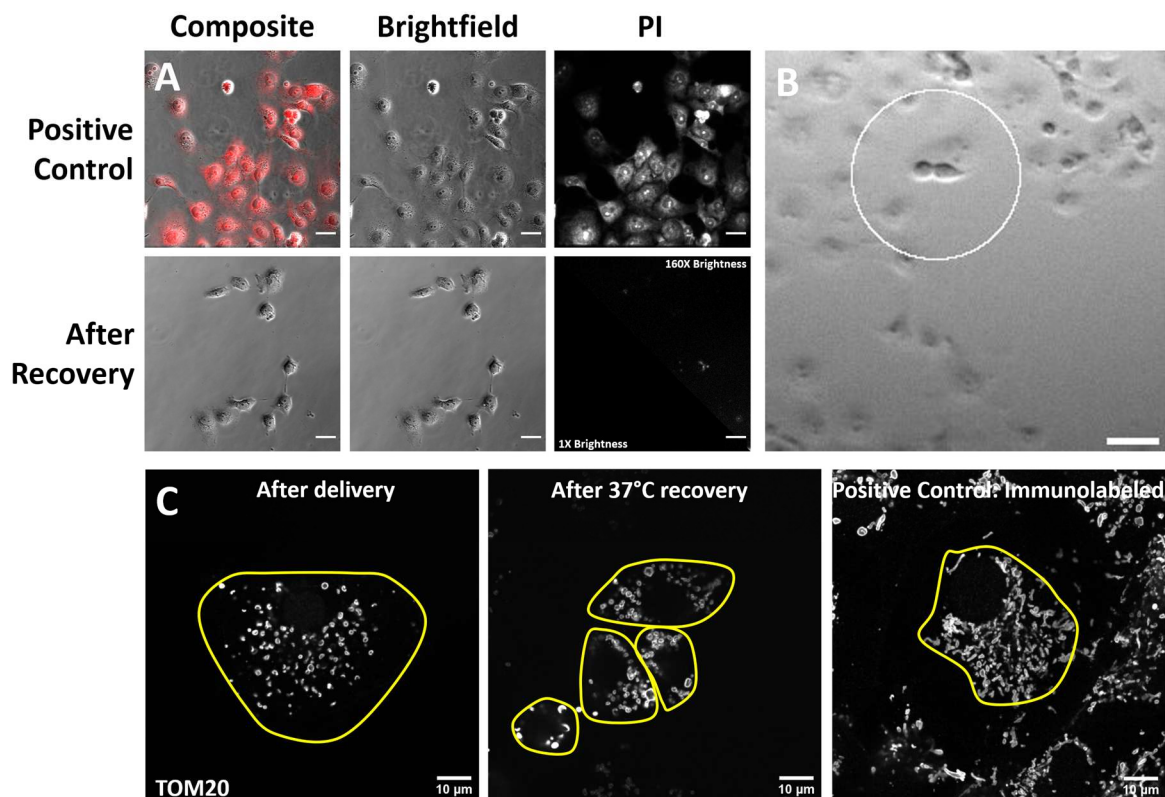


Fig. 3.2 Cell Membrane Recovers after a Prolonged Exposure to Saponin.*(a) After 30 minutes of incubation in complete growth media, cells become completely impermeable to bulky, charged nuclear stains (Propidium Iodide; PI) compared to a positive control with chemical induced membrane permeabilization. PI stain after recovery was imaged at the same brightness but no signal was detected; even at 160x brightness, no cells evidently remain permeabilized. (b) Membrane-recovered cells display healthy morphology and are capable of proliferating as indicated by the white circle. Live samples were imaged at 20x magnification. (c) Labeled mitochondria imaged after delivery and following recovery sequence. In both cases, similar labeling is observed to immunolabeled positive control. Scale bars = 10 μ m*

3.3.3 Validation of Nanogold probe conjugation and delivery

Although saponin-induced live-cell delivery was successful using fluorophore-conjugated antibody probes, nanogold-conjugated probes could introduce additional challenges due to their larger size and metal-label composition. To test whether this workflow could accommodate nanogold probes, I prepared fluorophore-nanogold dual-labeled secondary antibodies. The small-molecule fluorophore Atto647N and 5 nm nanogold were conjugated to donkey anti-rat and donkey anti-mouse secondary antibodies, and the resulting products were characterized using UV-Vis spectrophotometry (Fig. 3.3a). After confirming fluorophore and nanogold absorbance and optimizing dye-to-protein and nanogold-to-protein ratios, I delivered the dual-labeled probes intracellularly and observed fluorescence at expected target structures, including microtubules and the nuclear lamina. Silver enhancement was then used to amplify the nanogold signal for light microscopy visualization, confirming that the metal label was retained on the delivered probes and localized to the same target structures (Fig. 3.3b).

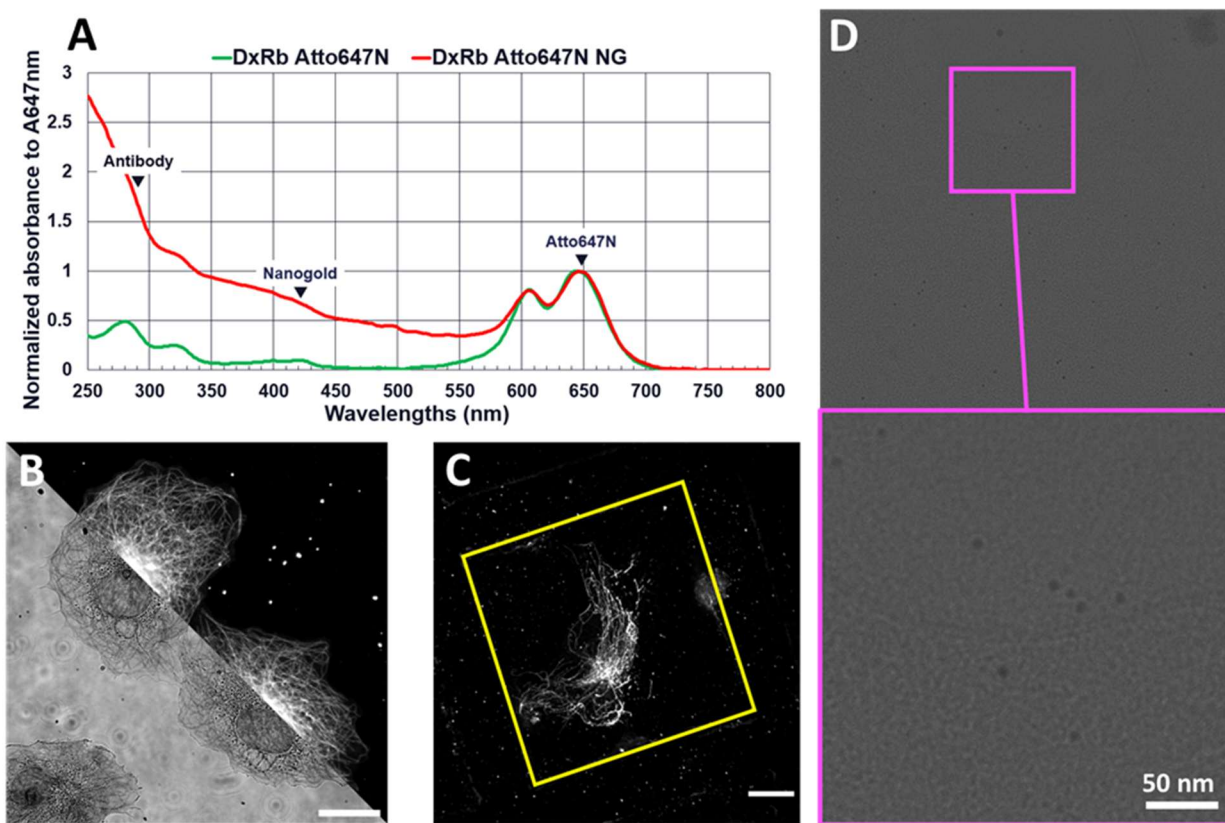


Fig. 3.3 Nanogold Conjugation to Antibody Probes and Detection by Optical and Electron Microscopy. (a) UV-vis absorbance spectrum of donkey anti-Rabbit secondary (DxRb) conjugated to Atto647N (green) and both Atto647N/Nanogold (red). Nanogold particles have a broad absorbance. Wavelengths used for calculation of antibody, Nanogold, and Atto647N are noted accordingly. (b) Phase contrast and fluorescence micrographs of DxRb Atto647N-NG delivered cells are overlaid to portray colocalization of silver-enhanced Nanogold and Atto647N. (c) Primary and multimodal secondary antibody probes are delivered to cells seeded on Quantifoil R 2/2 grid. Yellow box outlines a region of the mesh on the grid where the imaging is performed. Samples were fixed after live-cell delivery protocol. Fixed samples were imaged at 60x magnification. Scale bars = 20 μm. (d) Cryo-TEM image of 5 nm Nanogold (white arrow) on Quantifoil grid.

3.3.4 Preparation for electron microscopy applications

Cell culture on EM-compatible grids is well established, however to minimize reagent use, I devised a delivery method using a parafilm sheet with small dimples that could hold approximately 35 μ L of saponin, primary antibody, and secondary antibody solution directly over cells adhered to EM grids. After delivery and chemical fixation in initial tests, both fluorescence and silver-enhanced signals were observed at the expected microtubule and nuclear lamina structures (Fig. 3.3c). These results demonstrate that the saponin-based delivery workflow can be adapted to cells cultured on EM-compatible grids while preserving targeted labeling. Ongoing work by Yuka Kurasaka in the Vaughan group will extend this workflow to electron microscopy imaging, evaluate high-resolution localization of the labeled structures, and adapt the protocol for cryo-EM applications.

3.4 Methods

3.4.1 Transient cell membrane permeabilization and recovery

COS-7 cells were cultured in 8-well plates at a density of 300,000 cells per well 24 hours before delivery. Growth medium was removed from each well, and the cells were briefly washed with water. Saponin was added at 0.1 mg/mL to induce cell membrane permeabilization. Propidium iodide was added to one well of permeabilized cells at a final working concentration of 500 nM as a positive permeabilization control. After 10 minutes of incubation at room temperature, cells were washed with 1 $\times$ PBS (Thermo Fisher; 70011069) and fixed with either 3.5%

paraformaldehyde (PFA) and 0.5% glutaraldehyde (GA) in PEM buffer or 4% PFA for 10 minutes at room temperature. Cells were then washed with 1× PBS-azide for storage.

For the recovery condition, cells without propidium iodide were incubated in growth medium at 37 °C in a cell culture incubator. Following recovery, propidium iodide was added at the same working concentration to a separate well that had not been used for the positive control. Cells were incubated for 10 minutes at room temperature, washed and fixed as described above, and stored in 1× PBS-azide. Propidium iodide signal was imaged using 647 nm excitation.

3.4.2 Live-cell delivery of exogenous probes workflow

COS-7 cells were cultured in 8-well plates at a density of 300,000 cells per well 24 hours before delivery. Growth medium was removed from each well, and the cells were briefly washed with the appropriate incubation buffer for each antibody target: 1× PEM buffer containing 100 mM PIPES, 1 mM EGTA, and 1 mM MgCl₂ in water for anti-tubulin labeling, or water for anti-lamin A/C labeling. Cells were incubated with 0.1 mg/mL saponin for 10 minutes at room temperature to induce membrane permeabilization and then washed once with the corresponding incubation buffer.

Primary anti-tubulin antibody (Thermo Fisher; MA1-80017) or anti-lamin A/C antibody (Santa Cruz Biotechnology; sc-376248) was prepared at 350 nM in the respective incubation buffer. Saponin-permeabilized cells were incubated with primary antibody solution for 5 minutes at room temperature and then washed once with incubation buffer to remove unbound primary antibody. Atto488-conjugated donkey anti-rat antibody (Millipore Sigma; 41698-1MG-F) or

Alexa Fluor 647-conjugated donkey anti-mouse antibody (Lumiprobe; 26820) was prepared at 350 nM and incubated with the cells for 5 minutes at room temperature, followed by one wash with incubation buffer.

Cells were then fixed with either 3.5% paraformaldehyde (PFA) and 0.5% glutaraldehyde (GA) in PEM buffer or 4% PFA for 10 minutes at room temperature. After fixation, cells were washed with 1× PBS-azide and stored in 1× PBS-azide. Labeled cells were imaged using a spinning-disk confocal fluorescence microscope equipped with a 60×, 1.27 numerical aperture water-immersion objective.

3.4.3 Conjugation of fluorophore-Nanogold dual-labeled antibody probe

To prepare Atto647N-conjugated secondary antibodies, 12 µg of 1 mg/mL Atto647N-NHS ester in DMSO (Sigma-Aldrich; 18373-1MG-F) was incubated with 200 µL of 1.3 mg/mL donkey anti-rat antibody or donkey anti-mouse antibody, together with 50 µL of 1 M sodium bicarbonate (VWR Scientific; 470302-440). Donkey anti-rat antibody (Jackson ImmunoResearch; 715-005-153) and donkey anti-mouse antibody (Jackson ImmunoResearch; 715-005-151) were used for conjugation. Fluorophore-conjugated antibodies were separated from free dye using Amicon Ultra-4 centrifugal filter units with a 30 kDa molecular weight cutoff (MWCO; Millipore Sigma; UFC803024) and characterized for dye-to-protein ratio using a UV-Vis spectrophotometer (DeNovix DS-11+ Spectrophotometer).

The fluorophore-conjugated secondary antibodies were then conjugated to 5 nm nanogold (Nanoprobes Inc.; 2225A-5X1NMOL) according to the manufacturer's instructions. Free

nanogold was not removed after conjugation, and protein concentration was approximated based on the initial reagent amounts.

3.4.4 Cryo-EM

Quantifoil R 2/2 Au EM grids (Ted Pella Inc.; 657-200-AU) used for cell culture were plasma-cleaned for 1 minute and sterilized with 70% isopropyl alcohol. The grids were incubated with a 50× dilution of poly-L-lysine in water for 15 minutes at room temperature and then washed with water. Grids were transferred to a 35 mm micro-imaging dish, placed on the cover glass with poly-L-lysine droplets, and allowed to dry. Suspended COS-7 cells were plated onto the dish at a density of 200,000 cells/cm² one day before the experiment.

Live-cell delivery was performed directly on cell culture grids by inverting each grid onto droplets of the appropriate reagents. Probe-delivered cells were cryopreserved using liquid ethane and a custom grid plunge freezer. Cryo-electron tomography (cryo-ET) was performed using a Thermo Fisher Titan Krios G3 equipped with a Gatan BioQuantum Energy Filter and Gatan K3 Direct Electron Detector camera. Focused ion beam (FIB) milling was performed using a Thermo Fisher Aquilos II.

3.5 Conclusion and discussion

The work outlined in Chapter 3, which focuses on live-cell delivery of exogenous probes for cryo-EM labeling, remains ongoing. Although the main workflow from cell culture to cryofixation has been established, several challenges remain. The most prominent bottleneck is

the long turnaround time required for electron microscopy validation, which arises from both instrument operation and image-processing demands. Electron microscopy generally requires complex instrumentation, extensive user training, and access to limited shared resources. In addition, each image-acquisition session can span several days, substantially longer than a typical light-microscopy workflow for cell imaging. When combined with downstream image processing using specialized software, each experimental iteration can extend from weeks to months. This challenge is not specific to this project but reflects a broader limitation in electron microscopy research. With additional optimization and dedicated effort, the EM pipeline for this workflow could be further streamlined through continued iteration.

In conclusion, much of the live-cell delivery workflow has been established, with only minor additional optimization remaining. The next phase of this work will depend heavily on validation by electron microscopy. Key outstanding challenges include confirming nanogold detectability within complex biological backgrounds, quantifying labeling density at nanoscale resolution, and demonstrating clear, target-specific nanogold labeling following live-cell delivery in cryo-EM. Despite these challenges, this approach shows strong potential as a generalizable strategy for introducing targeted exogenous probes prior to cryofixation. With further optimization of delivery consistency, probe design, including smaller binders such as minibinders [15], and integration with other emerging EM labeling strategies, this method could provide a practical pathway toward targeted, multiplexed imaging in cryo-EM.

A few outstanding questions remain for this workflow and can be grouped into two major areas: robust and consistent live-cell delivery, and detection of nanogold labels by electron

microscopy. First, although saponin-induced membrane permeabilization shows strong and scalable potential for intracellular antibody delivery, delivery efficiency remains highly variable depending on cell-culture conditions, incubation buffers, and the physical technique used to transfer reagents to cells. Because the goal is to keep cells viable long enough for membrane integrity to recover during post-delivery incubation in growth medium, delivery efficiency must account not only for labeling of the target structure but also for cell survival after permeabilization.

Across many iterations of this experiment, a substantial fraction of cells grown in 8-well plates, frequently more than 80%, was lost during the delivery and wash steps, with only cells near the edges remaining adhered to the tissue-culture-treated glass bottom. Possible causes include differences in shear stress from pipetting at the center versus the edges of the well, as well as variability in user handling. Similar cell loss was observed for cells cultured on EM grids. Because grids are transferred directly to and from reagent droplets, this format introduces additional handling considerations, including shear stress as the grid is placed onto or removed from droplets. Saponin may also weaken membrane integrity enough to increase cell detachment from the substrate. Consistent with this possibility, lower saponin concentrations improved cell retention but reduced labeling efficiency, with approximately 50–80% labeling observed at 0.1 mg/mL and less than 20% labeling observed at 0.01 mg/mL. Therefore, further optimization is needed to balance saponin concentration, labeling efficiency, cell viability, and substrate-specific handling conditions.

The second, and more fundamental, question underlying this work is the detectability of nanogold in electron microscopy. Both 1.4 nm and 5 nm nanogold particles commercially available

from Nanoprobes Inc. have been validated for TEM detection, and we have verified that our instruments are capable of detecting nanogold by cryo-TEM. However, these tests were performed primarily using isolated nanogold in solution, without the obscuring background of biological samples. Although immunogold labeling of cells has been demonstrated in TEM, detection can be challenging and often appears most clearly in regions with concentrated labeling or nonspecific accumulation.

In this work, microtubules and the nuclear lamina were selected as target structures because they have recognizable ultrastructural features in TEM. The goal is to visualize sufficient nanogold labeling along these structures, as suggested by the fluorescence and silver-enhanced transmitted-light microscopy results. However, it is difficult to predict whether the labeling density observed by light microscopy is sufficient for nanogold detection by electron microscopy because of the diffraction-limited resolution of optical imaging. Labels spaced hundreds of nanometers apart could appear continuous by light microscopy while remaining sparse at the nanoscale resolution required for EM detection. Further evaluation of labeling density will therefore require multiple iterations of electron microscopy experiments and will remain constrained by the long turnaround time discussed above. More accessible super-resolution approaches, such as STORM or expansion microscopy, may help bridge this gap by providing an accessible assessment of labeling density before committing samples to electron microscopy.

3.6 References

1. A. Barski, S. Cuddapah, K. Cui, T.-Y. Roh, D. E. Schones, Z. Wang, G. Wei, I. Chepelev, and K. Zhao, "High-Resolution Profiling of Histone Methylations in the Human Genome," *Cell* **129**(4), 823–837 (2007).
2. E. Lieberman-Aiden, N. L. van Berkum, L. Williams, M. Imakaev, T. Ragozy, A. Telling, I. Amit, B. R. Lajoie, P. J. Sabo, M. O. Dorschner, R. Sandstrom, B. Bernstein, M.

- A. Bender, M. Groudine, A. Gnirke, J. Stamatoyannopoulos, L. A. Mirny, E. S. Lander, and J. Dekker, "Comprehensive Mapping of Long-Range Interactions Reveals Folding Principles of the Human Genome," *Science* **326**(5950), 289–293 (2009).
3. Y. Liu, C. D. McGann, C. P. Herlihy, M. Krebs, T. A. Perkins, R. Fields, C. K. Camplisson, D. Z. Nwizugbo, Q. Lin, N. J. Longhi, C. Hsu, S. C. Avanessian, A. F. Tsue, E. E. Kania, D. M. Shechner, B. J. Beliveau, and D. K. Schweppe, "DNA O-MAP uncovers the molecular neighborhoods associated with specific genomic loci," (2026).
4. J. B. Harris, T. G. Williams, and J. A. Schultz, "Simplified heavy metal staining techniques demonstrated with Fast Plant leaf tissue," *Cell Res.* **2**(1), 89–96 (1992).
5. J. F. Hainfeld and F. R. Furuya, "A 1.4-nm gold cluster covalently attached to antibodies improves immunolabeling," *J. Histochem. Cytochem.* **40**(2), 177–184 (1992).
6. Y. Hsia, J. B. Bale, S. Gonen, D. Shi, W. Sheffler, K. K. Fong, U. Nattermann, C. Xu, P.-S. Huang, R. Ravichandran, S. Yi, T. N. Davis, T. Gonen, N. P. King, and D. Baker, "Design of a hyperstable 60-subunit protein icosahedron," *Nature* **535**(7610), 136–139 (2016).
7. M. H. Laporte, N. Klena, V. Hamel, and P. Guichard, "Visualizing the native cellular organization by coupling cryofixation with expansion microscopy (Cryo-ExM)," *Nat. Methods* 1–7 (2022).
8. M. Wassler, I. Jonasson, R. Persson, and E. Fries, "Differential permeabilization of membranes by saponin treatment of isolated rat hepatocytes. Release of secretory proteins," *Biochem. J.* **247**(2), 407–415 (1987).
9. K. Medepalli, B. W. Alphenaar, R. S. Keynton, and P. Sethu, "A new technique for reversible permeabilization of live cells for intracellular delivery of quantum dots," *Nanotechnology* **24**(20), 205101 (2013).
10. F. Cardarelli, L. Digiacomo, C. Marchini, A. Amici, F. Salomone, G. Fiume, A. Rossetta, E. Gratton, D. Pozzi, and G. Caracciolo, "The intracellular trafficking mechanism of Lipofectamine-based transfection reagents and its implication for gene delivery," *Sci. Rep.* **6**(1), 25879 (2016).
11. S. Hennig, S. van de Linde, M. Lummer, M. Simonis, T. Huser, and M. Sauer, "Instant Live-Cell Super-Resolution Imaging of Cellular Structures by Nano-injection of Fluorescent Probes," *Nano Lett.* **15**(2), 1374–1381 (2015).
12. A. Sharei, J. Zoldan, A. Adamo, W. Y. Sim, N. Cho, E. Jackson, S. Mao, S. Schneider, M.-J. Han, A. Lytton-Jean, P. A. Basto, S. Jhunjhunwala, J. Lee, D. A. Heller, J. W. Kang, G. C. Hartoularos, K.-S. Kim, D. G. Anderson, R. Langer, and K. F. Jensen, "A vector-free microfluidic platform for intracellular delivery," *Proc. Natl. Acad. Sci.* **110**(6), 2082–2087 (2013).
13. C. J. McKinlay, J. R. Vargas, T. R. Blake, J. W. Hardy, M. Kanada, C. H. Contag, P. A. Wender, and R. M. Waymouth, "Charge-altering releasable transporters (CARTs) for the delivery and release of mRNA in living animals," *Proc. Natl. Acad. Sci.* **114**(4), (2017).

14. Y.-C. Wu, T.-H. Wu, D. L. Clemens, B.-Y. Lee, X. Wen, M. A. Horwitz, M. A. Teitell, and P.-Y. Chiou, "Massively parallel delivery of large cargo into mammalian cells with light pulses," *Nat. Methods* (2015).
15. L. Cao, I. Goresnik, B. Coventry, J. B. Case, L. Miller, L. Kozodoy, R. E. Chen, L. Carter, A. C. Walls, Y.-J. Park, E.-M. Strauch, L. Stewart, M. S. Diamond, D. Veessler, and D. Baker, "De novo design of picomolar SARS-CoV-2 miniprotein inhibitors," *Science* **370**(6515), 426–431 (2020).

Chapter 4

Future Directions

4.1 Overview

As presented in this dissertation, I developed two multiplexed imaging strategies for spectral fluorescence microscopy and electron microscopy that could provide complementary visualization of biological systems. In Chapter 2, I introduced a rapid and accessible spectral imaging method that concurrently detects 11 biomarkers with single excitation and acquisition. In Chapter 3, I discussed the optimization of a workflow involving saponin-induced transient cell membrane permeabilization and live-cell delivery of antibody probes for cryo-EM workflows. In this chapter, I will briefly discuss broader future directions for both strategies to improve their capabilities and applications.

4.2 Future directions for SLiM

Multiplexed biomarker detection is highly desirable in both research and clinical settings because it can reduce the number of experimental iterations needed to evaluate spatial relationships and colocalization profiles. Increasing the number of simultaneously detectable targets from the previously achievable six to eight channels to eleven channels reduces the number of experiments needed to probe pairwise relationships among eleven biomarkers by approximately 3- to 6-fold. In addition, reduced acquisition time and workflow automation for image processing and spectral unmixing could support adoption into day-to-day pathology workflows by improving turnaround time from biopsy to diagnosis. Therefore, future work

should test the feasibility of adapting the SLiM system to platforms more comparable to commercially available instruments used in pathology. To support this translation, the sensitivity of the SLiM system should be further investigated with additional probe panels, including small-molecule fluorophores and quantum dots, as well as tissue samples more commonly used in clinical settings.

Extending the SLiM concept through careful calibration of the existing adjustable slit could also provide an opportunity for multiplexed volumetric imaging. Currently, the slit opening is set at approximately 280 μm . With custom or commercial slits capable of producing smaller openings, SLiM may be adapted for confocal imaging through thicker tissue volumes. Because the SLiM control software already determines scan positions automatically based on initial imaging parameters, and because the system includes a piezo Z-axis stage, the current configuration could be extended toward rapid, multiplexed volumetric imaging with minimal switching of optical components or recalibration.

4.3 Future directions for live-cell delivery and cryo-EM labeling

The biggest advantage of the live-cell delivery workflow introduced in Chapter 3 is its simplicity and accessibility, which could allow it to be adapted to other existing protocols without incorporating extensive or time-consuming additional steps. The use of saponin for live-cell delivery does not require specialized equipment, and the permeabilization can be reversed to support recovery of cell function, such as proliferation. First and foremost, the most direct future application of this workflow is its continued development for cryo-EM workflows, where labeling strategies remain limited and often require multiple reagent exchanges. Another potential application is live-cell or in vitro labeling and imaging, where reversible

permeabilization may reduce perturbation of vesicles, the nuclear membrane, or other membrane-bound structures while enabling delivery of probes against macromolecular targets that are difficult to access using other delivery techniques.

Another direction for extending this live-cell delivery workflow is to introduce multiplexed probe sets, where two or more probe types are delivered and labeled in the same round to test their feasibility. Furthermore, this workflow could potentially be used to introduce materials other than fluorescent antibody probes, such as labeled tubulin, CRISPR-associated reagents, or drug-conjugated proteins, to evaluate its broader utility beyond diagnostic imaging applications.