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BY
DAOZHENG GONG

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Dedicated to my family and friends

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ABSTRACT

Structured Illumination Microscopy (SIM) is gaining increasing attention in the field of bioimaging for its capability to strike a balance between high spatial resolution and fast imaging speed. However, adopting SIM in high-dimensional imaging faces various challenges, including aberrations, limited color channel availability and the long acquisition time for a whole high-dimensional data cube. Here, a novel snapshot multi-dimensional structured illumination microscopy (SIM) imaging system is devised to address these issues and achieves the following: (1) Aberration-corrected super-Resolution Imaging: We introduce a hybrid adaptive optics system, TACO, correcting both illumination and excitation aberrations. This results in high-quality SIM super-resolution imaging even in highly aberrative environments. (2) Scalable multi-color Imaging: A dispersion compensation grating module was designed to correct the divergence of different excitation wavelength beams, enabling effortless increase in the number of color channels with minimal realignment. (3) Multi-scan 3D Imaging: Simultaneous multiplane imaging is achieved through a specialized multi-plane beam splitter. A deformable mirror facilitates rapid z-scan and aberration correction, ensuring fast, aberration-free volumetric imaging. (4) Virtual multi-channel imaging: Our system adapts a convolutional neural network model with a novel architecture for automated decomposition of multiple cellular structures in a single channel. (5) Single-Shot SIM super-resolution reconstruction: a novel SIM reconstruction algorithm is developed to enable super-resolution reconstruction from one snapshot by encoding multiple image frames through compressive patterns. The presented SIM system’s capabilities have been rigorously validated through experimental imaging or computational simulation. These innovations significantly broaden the application of SIM in complex high-dimensional data acquisition tasks.

CHAPTER 1

INTRODUCTION

1.1 Optical microscopy and super-resolution imaging

1.1.1 *Optical microscopy*

The invention of optical microscopy is one of the most significant breakthrough of biology, and the development of "super-resolution" techniques represents one of the most thrilling recent and ongoing advances in microscopy. Approximately 400 years ago, Robert Hooke and Antonie van Leeuwenhoek began the study of organisms at the microscale using optical microscopes they developed themselves (Cole (1939); Gest (2004)). Since then, optical imaging has served as the workhorse for investigating the structures and dynamics of biological systems and in medical research. In 1873, about 200 years later, Ernst Abbe discovered that the resolution of optical microscopy is physically limited by the diffraction of light (Abbe (1873)). This resolution limit, also referred to as the Abbe diffraction limit, indicating the minimum feature size d that can be measured is given by:

$$d = \frac{\lambda}{2n \sin(\theta)}, \quad (1.1)$$

where λ is the wavelength of the light, n is the index of refraction of the imaging medium, and θ is the half-angle of the maximum cone of light that can enter the objective lens. The numerical aperture of a microscopy system is typically defined as $NA = n \sin(\theta)$, allowing the diffraction limit to be expressed as $d = \lambda/2NA$.

As a consequence of the diffraction limit, the resolution of conventional optical microscopy is limited to approximately 200 nm, which substantially hinders its application for observing biological structures and events at the nanometer scale. Fortunately, rather than a fundamental law of nature, the diffraction limit is more accurately described as an empirical

feature of a lens system (Astratov et al. (2023)). Upon closer examination, it becomes evident that there are numerous practical assumptions necessary for the Abbe diffraction limit to apply:

- assume that the sample is densely distributed or labeled;
- assume that the illumination light is uniform;
- assume that the signal is linearly responsive to the illumination;
- assume that no evanescent fields are detected;
- assume that the signal is temporally stable;
- assume that the light propagates as classic waves;
- ...

150 years after the establishment of the Abbe diffraction limit, it was recognized that these assumptions do not necessarily have to hold true in an optical system. Consequently, an increasing number of techniques have been developed to "break" the diffraction limit by challenging one or more of these assumptions, thereby achieving what is termed "super-resolution" imaging. This innovation has ushered in a new era of microscopy imaging in the 21st century.

1.1.2 Super-resolution imaging

When a sample is sparsely distributed or labeled, such as a sparsely distributed fluorescent nanobeads, the emission from each can be modeled using a Gaussian distribution, allowing the bead's localization to be determined with precision far exceeding the diffraction limit (Thompson et al. (2002); Qu et al. (2004)). The localization precision can be approximated

as:

$$\Delta_{SMLM} = \frac{\Delta_{Abbe}}{\sqrt{N}}, \quad (1.2)$$

where Δ_{SMLM} and Δ_{Abbe} are the localizations determined by Gaussian fitting and the Abbe diffraction limit, respectively, and N is the number of collected photons. As the equation shows, given a sufficiently large number of photons, the localization precision is theoretically unlimited. However, in typical biological experiments, samples are usually densely distributed or labeled with continuous shapes, making it impossible to directly fit individual fluorescent points.

Single molecule localization microscopy (SMLM) is a class of super-resolution techniques that addresses this limitation by imaging only a subset of "blinking" labels at a time with high precision. Photoactivated localization microscopy (PALM, Betzig et al. (2006)) and stochastic optical reconstruction microscopy (STORM, Rust et al. (2006)) are two representative SMLM methods that utilize photoactivatable fluorescent labels which can be switched on and off by illumination with a different wavelengths of light. During each imaging exposure, only a small portion of the labels are stochastically activated, thus artificially creating a sparsely distributed sample allowing precise localization of each fluorophore. Typically, thousands of images are required to reconstruct a complete field of view with enough localized points to enable structural identification. Each single fluorescent molecular label can emit sufficient detected photons ($O(10^3)$) to obtain localization resolution of ~ 20 nm.

Apart from using photoactivatable fluorescent labels, another method to create "blinking" is through the stochastic binding of labels via diffusion. DNA points accumulation for imaging in nanoscale topography (DNA-PAINT, Jungmann et al. (2010, 2014)) utilizes the transient binding of freely floating short DNA oligonucleotides (imager strands) with targets labeled with complementary DNA oligonucleotides (docking strands) to achieve blinking similar to that in STORM/PALM. Recently, a variant of resolution enhancement by sequential imaging (RESI, Reinhardt et al. (2023)) in DNA-PAINT that uses more than one

orthogonal DNA barcode to sequentially label different subsets of a single target species has been developed. This sequential labeling divides the targeted sample into even sparser subsets, thus enhancing sparsity beyond that achieved solely through DNA diffusion. This technique advances the resolution of SMLM to the Ångström scale, enabling the resolution of extremely fine structures, such as the eight pairs of single Nup96 proteins that form the eight-fold symmetric structure of the nuclear pore complex, previously only observable by techniques such as cryo-electron microscopy.

A non-uniform distribution of illumination intensity can also be leveraged to achieve super-resolution imaging. Structured Illumination Microscopy (SIM) utilizes patterned illumination, typically a sinusoidal pattern, to encode high-resolution information of a sample into low-resolution patterns that can be captured by the optical system. The high-resolution information is then recovered through computational reconstruction (Gustafsson (2000); Gustafsson et al. (2008)). In the case of sinusoidal patterns, SIM typically requires three sets of illumination patterns at different orientations, with each orientation set having 3-5 phase shifted images. This results in 9-15 images in total being required for reconstructing a super-resolution image. Conventional SIM can enhance resolution by up to a factor of two. This limited enhancement is due to the fact that the illumination pattern itself is diffraction-limited, and the finest period of the pattern cannot be smaller than the diffraction limit. To further surpass the resolution limits of SIM, nonlinear-SIM has been developed by challenging both the uniform illumination assumption and the linear response assumption (Gustafsson (2005); Heintzmann and Gustafsson (2009)). This method utilizes very high-intensity illumination to saturate the fluorescent labels, achieving illumination patterns with even higher spatial frequency. Theoretically, nonlinear-SIM can achieve arbitrarily high resolution. Although standard SIM may not provide as significant a resolution enhancement as other super-resolution techniques, it is much faster and only requires modest conditions for the labels and illumination intensity, making it the most suitable technique for imaging live

organisms Zhao et al. (2021). Structured illumination is the main topic of my dissertation, and a detailed discussion of the principles of SIM is presented in Chapter 2.

Stimulated Emission Depletion (STED, by Klar et al. (2000)) represents another approach to achieving super-resolution by combining non-uniform illumination with a nonlinear response. In addition to a conventional Gaussian illumination beam, STED employs an extra donut-shaped depletion beam of a different wavelength that forces excited fluorophores back to a non-fluorescent state through stimulated emission. Consequently, only the fluorophores in the center of the donut can still emit fluorescences, resulting in an emission distribution significantly narrower than the diffraction limit. The resolution achieved by STED can be theoretically approximated as:

$$\Delta_{STED} = \frac{\Delta_{Abbe}}{\sqrt{1 + I/I_0}}, \quad (1.3)$$

where Δ_{STED} and Δ_{Abbe} represent the localizations determined by STED and the Abbe diffraction limit, respectively. I is the peak intensity of the depletion beam, and I_0 is the threshold intensity for stimulated emission. STED can typically achieve resolutions around ~ 20 nm. Despite its comparable performance to SMLM, STED requires much higher illumination intensity to effectively suppress fluorescence in the donut area, leading to significant phototoxicity and limiting its application. Reversible Saturable Optical Fluorescence Transitions (RESOLFT, by Hofmann et al. (2005)) employs a similar concept with a donut-shaped beam but uses photo-switchable fluorophores rather than stimulated depletion to suppress the fluorescence, making it more compatible with live organism imaging. Also based on the donut-shaped beam concept, Minimal Emission Fluxes Microscopy (MINFLUX, by Balzarotti et al. (2017)) and its variant MINSTED (Weber et al. (2022)) achieve unprecedented resolutions of 1 nm, or even ångström scale, by repeatedly scanning the donut beam around a target fluorescent molecule. Here, the donut beam serves as an excitation rather than suppression mechanism; when the fluorescent molecule co-localizes

with the center of the donut beam, the fluorescence signal reaches its minimum. Compared to previous super-resolution techniques such as SMLM and STED, MINFLUX represents a significant leap in resolution from ~ 20 nm to sub-nanometers, potentially initiating a new era where optical microscopy can be used to resolve protein structures previously only accessible by electron microscopy (Grabner et al. (2022)) or to investigate molecule interactions at a sub-nanometer scale, inaccessible by other techniques such as single-molecule FRET (Lerner et al. (2018)). Moreover, unlike other techniques that rely on a large number of collected photons for improved resolution, MINFLUX requires a minimum number of photons for high precision localization, making it particularly suitable for long-time imaging of small molecules (Scheiderer et al. (2023)).

The invention of MINFLUX initiated a new wave of super-resolution techniques that embrace a similar philosophy, including ROSE (Gu et al. (2019)), SIMFLUX (Cnossen et al. (2020)), RASTMIN (Masullo et al. (2022)), and ModLoc (Jouchet et al. (2021)). These methods utilize patterned illumination to achieve resolutions surpassing those of "traditional" super-resolution techniques like SMLM and STED. While they may not reach the extremely high resolutions of MINFLUX/MINSTED, they generally offer higher imaging speeds, thus achieving a better balance between spatial and temporal resolution. This makes them potentially more suitable for imaging over large fields of view (FOV).

There are many other super-resolution techniques that challenge the assumptions underlying the Abbe diffraction limit. Super-resolution Optical Fluctuation Imaging (SOFI, by Dedecker et al. (2012)) produces super-resolution images by detecting the temporal fluctuations of fluorophores and extracting additional information through statistical analysis. Beyond microscopy techniques based on "classic" optics, a new class of super-resolution methods that exploit the "quantum" properties of photons is emerging and gaining interest (Israel et al. (2017); Tenne et al. (2019); Classen et al. (2017)). These techniques utilize information from photon correlation among single-photon emitters to determine the number

of active emitters within a finite area with enhanced precision compared to intensity measurements, typically by a factor of 2. Although proof-of-principle demonstrations have been shown, applying these techniques to real biological research remains challenging due to the complexities of correlation measurements.

It's important to note that the list of assumptions for the Abbe diffraction limit is not intended to be exhaustive; more assumptions that might seem trivial could prove to be unexpectedly powerful when appropriately leveraged. For example, expansion microscopy negates the assumption that the physical size of the sample is fixed, achieving nanometer resolution by physically expanding a sample using hydrogel techniques (Chen et al. (2015); Shaib et al. (2023)). Typically, a sample can be expanded by a factor of 4, leading to approximately 60 nm resolution with conventional wide-field microscopy. Iterative expansion microscopy (Chang et al. (2017); Louvel et al. (2023)) has also been developed to iteratively expand a sample, achieving even higher expansion factors that could reach up to 20. While expansion microscopy has the apparent limitation of being unsuitable for imaging live organisms, it demonstrates exceptionally powerful performance when applied to research where live imaging can be compromised, such as in revealing the nanostructures of proteins (Shaib et al. (2023); Kyllies et al. (2023)) or mapping the spatial distribution of molecules among tissues (Klimas et al. (2023); Cui et al. (2023)).

Despite the impressive progress of super-resolution techniques, it's crucial to emphasize that resolution is not the only important parameter of a microscope. In fact, achieving ultra-high resolution often comes with significant trade-offs in other aspects of acquiring information about the sample of interest, which can lead to serious drawbacks in achieving the research goals. For instance, the extremely long waiting times required for SMLM or MINFLUX to reconstruct a high-resolution image make it nearly impossible to conduct time series imaging with a field of view relevant for cell biology. Expansion microscopy requires terminating the sample before imaging. Eric Betzig, the inventor of PALM, expressed his

concerns about the excessive focus on resolution during a recent talk: "People still seem to have in their head this idea that the metric that matters for a microscope is its resolution. Resolution is one metric for a microscope, but speed, non-invasiveness, all these other things are equally important for discovery." (Betzig, Eric (2024), Confession of a Frustrated Optical Microscopist). Clearly, impactful biological research requires a balance between resolution and many other factors, which will be discussed in the next section and is the main focus of this dissertation as well.

1.2 Imaging in high dimensions: volume, color, and time

Apart from resolution, there are numerous aspects of microscopy imaging that are equally or even more important. One of the most crucial aspects is the capability to conduct high-dimensional data recording, rather than just capturing high-resolution 2D images. A typical 5D dataset includes 3D spatial coordinates (xyz), multiple colors (c), and time series (t), collectively referred to as xyzct. Each of these dimensions poses unique challenges for the microscopy system.

To acquire high-quality 3D data, the imaging system must eliminate the effects of out-of-focus light or even enhance resolution along the z-axis. Furthermore, when imaging particularly thick tissues, such as brain or tumor samples, robustness against sample-induced scattering and aberrations becomes essential.

For multi-color data acquisition, the system needs to effectively resolve emission signals from different targets and prevent crosstalk between them. This challenge escalates as the number of targets increases.

Time series imaging is usually straightforward since most modern detectors provide video recording functions. However, as the time required to obtain each frame of progressively higher resolution, larger 3D volume, and more color channel images increases, therefore maintaining an acceptable frame rate to observe dynamics becomes a new challenge.

In fact, there are always trade-offs between different aspects of data acquisition in an imaging system; improving one aspect often leads to degradation in another. This difficulty in finding an optimal balance among all aspects is sometimes referred to as the "frustration pyramid" (Fig. 1.1).

In addition to resolution and dimensionality, another crucial factor is the signal-to-noise ratio (SNR) of the images. Obtaining adequate SNR is arguably the most important factor for imaging while also being the most compromised. For instance, in 3D imaging, much of the signal is discarded to filter out out-of-focus light. Similarly, for multicolor imaging, fluorescence filters with narrow bandwidths are used to distinguish different types of fluorophores, but this also leads to the loss of signal from target fluorophores whose emission falls outside the filter range. Moreover, imaging speed is inversely proportional to SNR, i.e., increasing the frame rate inevitably decreases the amount of signal collected in each frame. SNR often increases with $\sqrt{N}$ for N detected photons in a given pixel of an array detector. Also, there are sources of background noise (e.g., thermal versus photo-electrons) and read-out noise (from analog to digital converters and amplifiers) that are a constant that must be overcome to have $SNR > 1$.

Much of the effort in microscopy research involves trying to expand one vertex of the "frustration" pyramid while minimizing the shrinkage of others. New approaches for optimization of all aspects of the pyramid is the main focus of my dissertation. The remainder of this section provides a brief overview of the issues that need to be addressed for high-dimensional imaging.

1.2.1 Volumetric imaging

Optical sectioning is the most crucial prerequisite for conducting meaningful volumetric imaging (Dan et al. (2013)). An image with effective optical sectioning means that most of the signal presented in the image originates from a relatively narrow z-range around the focal

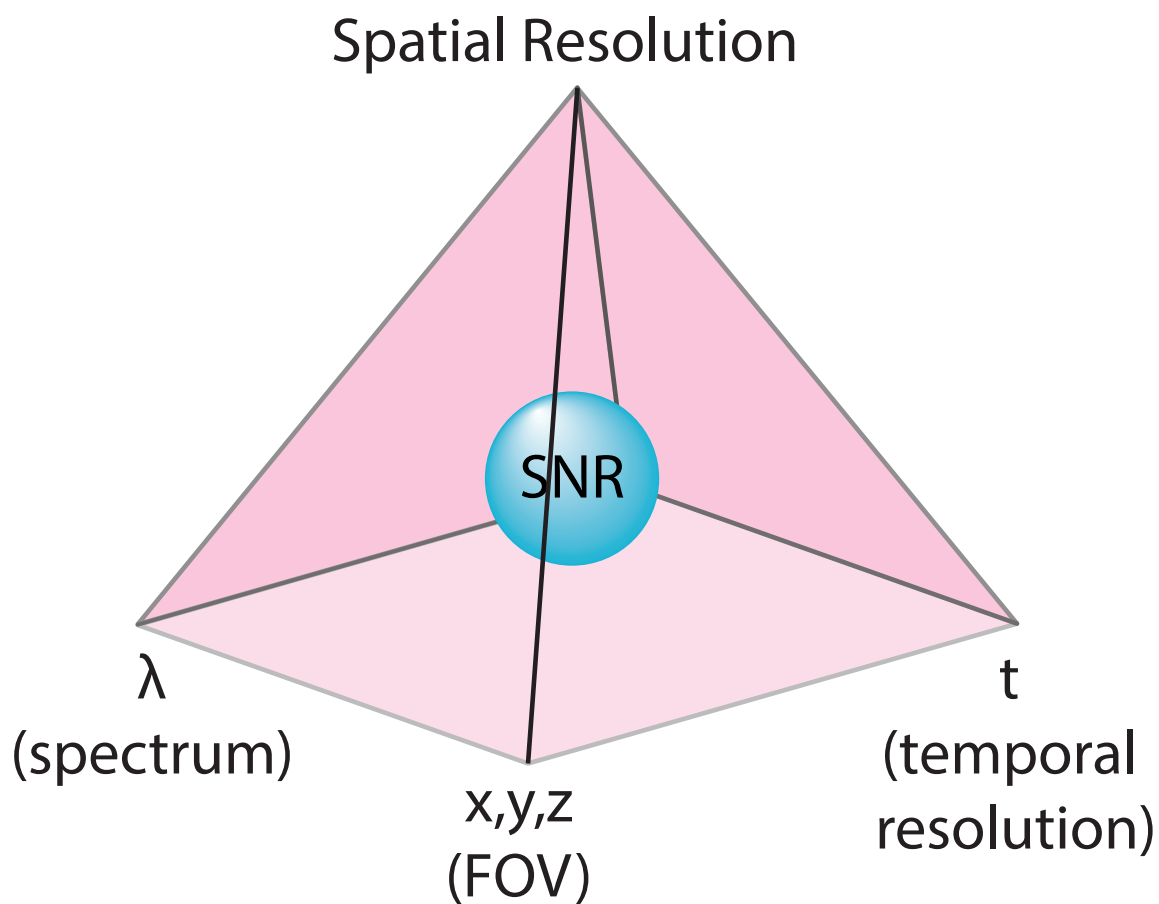


Figure 1.1: When designing a microscopy system, it is crucial to consider multiple parameters, as an improvement in one parameter often inevitably leads to a frustrating deterioration in others.

plane, while the signal emitted far from the focal plane is mostly rejected. Optical sectioning allows the correlation of images taken at one focal plane with a specific slice of the sample. A 3D image can then be reconstructed by taking a series of images that scan the focal plane through multiple planes of the sample, i.e., to obtain a 3D volume from "z-sclices".

Conventional wide-field microscopy illuminates the entire volume of the sample and collects signals throughout the sample. Although only signals near the focal plane can form sharp images, signals from other planes contribute a strong background making it impossible to separate information from individual focal planes effectively. This out-of-focus signal is another type of background noise that can obscure finer details and degrade the overall quality and usability of the 3D image data.

One effective way to achieve optical sectioning is by optically truncating the out-of-focus signal. Confocal microscopy (Minsky (1988); Pawley (2006)), uses a point detector to scan through an xy field of view, with a pinhole placed in front of the detector to physically block out-of-focus light from arriving at the actual detector. This method is highly effective in achieving bioimaging with a clean background and has arguably become the most widely used microscopy technique. However, the point-scanning configuration limits the speed of confocal microscopy. Spinning disk confocal microscopy (Pawley (2006)) addresses this limitation by using an array of moving pinholes on a disk to simultaneously image multiple points onto an array detector, significantly increasing the speed of acquiring a full field of view image. Image Scanning Microscopy (ISM, Müller and Enderlein (2010)) eliminates the physical pinhole and utilizes "digital pinholes" through image processing instead, achieving similar optical sectioning performance. These methods allow for enhanced imaging speed and flexibility while maintaining effective sectioning capabilities.

Structured Illumination Microscopy (SIM) represents another approach to achieving optical sectioning by using patterned illumination with a modulated intensity distribution not only in the lateral direction but also axially (Gustafsson et al. (2008)). Compared to confocal

microscopy, SIM requires only a few images with different patterns to reconstruct an optical-sectioning image, which can significantly enhance imaging speed. Detailed discussions of SIM for imaging 3D samples are presented in Chapters 2 and 4 of the dissertation.

Despite the successful optical sectioning performance of confocal and SIM, one significant limitation is their low signal efficiency. When collecting signals from one focal plane, the entire volume of the sample must be illuminated, and most of the signal is discarded, which can lead to significant side effects such as photobleaching. A method to improve this is to shift from detecting only the focal plane to illuminating only the focal plane, leading to the invention of light sheet microscopy (Voie et al. (1993); Stelzer et al. (2021)). Unlike traditional approaches that use one objective lens for both illumination and detection, light sheet microscopy typically employs two orthogonally placed objectives, one for illumination and one for detection. As the name suggests, the illumination beam is modulated into a thin sheet spreading along one direction while being focused along another. Since the illumination propagates perpendicular to the detection path, only the slice of the sample illuminated by the "light sheet" is imaged by the detection path while all other parts of the sample remain dark, thus achieving effective optical sectioning. Beyond the advantage of reduced photobleaching, light sheet microscopy is exceptionally user-friendly (arguably even more so than confocal microscopy, as no xy scanning of the sample is needed). Moreover, the decoupling of illumination and detection via separate optical paths makes it very flexible and customizable for various applications.

Although there is no comprehensive study, it is very likely that light sheet microscopy represents the microscopy technique with the most variants to date. One significant variation is the single-objective light sheet (Voleti et al. (2019); Yang et al. (2019); Sapoznik et al. (2020)), which uses only one objective for both light sheet generation and fluorescence detection. Eliminating the second objective addresses one of the major obstacles in light sheet microscopy: the limited space available for mounting samples. The advancements

in single-objective light sheet microscopy significantly expand its applications, making it a highly promising candidate to supersede confocal imaging. Another important class of light sheet variants includes techniques that combine light sheet microscopy with super-resolution methods, such as structured illumination (Chen et al. (2014, 2022)), RESOLFT (Bodén et al. (2024)), and expansion microscopy (Glaser et al. (2023)). Benefiting from the simplicity of light sheet technology, it is naturally convenient to integrate light sheet imaging with other existing imaging techniques, making it a nearly universal solution for achieving optical sectioning.

Light sheet microscopy has demonstrated exceptional performance when imaging cells and small transparent organisms such as worms or zebrafish. However, its effectiveness diminishes with strongly scattering tissues like brain or tumor tissues due to the limited penetration depth of light through these materials. To overcome this limitation and image deeper into tissues, two-photon or multiphoton microscopy has been developed (Denk et al. (1990); So et al. (2000)). In two-photon microscopy, fluorophores simultaneously absorb two long-wavelength photons to emit one fluorescence photon. This mechanism allows for fluorescent excitation using much longer wavelengths of excitation light, which are less affected by scattering (e.g. Rayleigh scattering scales as $1/\lambda^4$) and can therefore penetrate deeper into tissues. Additionally, three- and four-photon microscopy have been demonstrated, utilizing even longer wavelength excitation for deeper tissue imaging (Murakoshi et al. (2023)).

Apart from optical sectioning, another crucial issue that must be considered when conducting 3D volumetric imaging is aberration. As light propagates through the imaging medium and the samples, the wavefront of the light is likely to be disrupted due to factors such as optical misalignment, sample heterogeneity, and refractive index mismatch, leading to degradation of image quality. Adaptive optics (Ji et al. (2010); Ji (2017); Booth et al. (2002)) is a rapidly growing field that employs active optical components, which have been used for > 40 years in large telescopes and astronomy (Rodier (1981)). These active optical

devices, such as deformable mirrors and spatial light modulators, allow wavefront shaping in response to existing wavefront distortions. By optimizing the parameters of the wavefront shaper, aberrations can be counteracted and the image quality can be largely restored. A detailed discussion about adaptive optics can be found in Chapter 3.

1.2.2 Multi-color imaging

Color, or the emission spectrum, is another vital dimension in microscopy imaging. Unlike real-life color photography where the colors of different objects are intrinsic properties determined by their materials, the colors in microscopy imaging arise from the emission spectra of various fluorescent labels Pawley (2006). These labels can be customized and engineered to best meet the specific needs of the imaging application. An ideal multi-color microscopy image may contain multiple distinct color channels, each conveying the spatial information of one target imaging species. This can be achieved by selecting fluorescent labels with well-separated spectra and sequentially stimulating each label, ensuring that only one target species is imaged during each stimulation. This method facilitates clear differentiation between different biological structures or components within a sample.

Just as 3D imaging requires the prevention of signal crosstalk from different z-slices, a critical issue in multi-color imaging is avoiding the crosstalk between different color channels that arises from spectrum overlap among various labels (Friedl et al. (2023)). Fortunately, compared to 3D imaging, this color channel crosstalk can usually be straightforwardly resolved by implementing bandwidth filters to narrow the detectable wavelength ranges. While some signal is lost in this process, this method generally performs well when imaging only a few color channels. However, when it is necessary to image a larger number of targets, finding fluorescent labels with distinct and minimally overlapping emission spectra becomes much more challenging. In such cases, it is insufficient to represent each fluorescent label with a single color, such as "green" or "red". Instead, a detailed inspection of each label's

emission spectrum is essential to extract more information to differentiate them.

The images recorded with crosstalk between different color channels can be treated as linear combinations of the spectra of different labels, and the true distribution of each label can be reconstructed from prior knowledge of their emission spectra. This computational method is generally referred to as "spectrum demixing" (Wu et al. (2023); Bruggeman et al. (2023); Wang et al. (2023)) and is discussed in more detail in Chapter 6. This approach allows for more precise separation and identification of multiple targets within a single sample, enhancing the clarity and utility of multi-color microscopy images.

Another method to better resolve different colored labels involves directly recording the emission spectrum by integrating a spectrometer with the microscopy setup, a technique known as hyperspectral imaging (Gao and Smith (2015)). One of the simplest and most classic configurations is to place a prism in the detection path of a point-scanning microscope, such as a confocal microscope, and replace the point detector with a line detector to record the spectrum distribution (Sinclair et al. (2006)). Hyperspectral imaging directly provides a high-dimensional data cube and offers robust information for separating different colored labels. This approach enables precise spectral analysis of each pixel in the image, facilitating the distinction and quantification of multiple fluorophores within a single sample.

1.2.3 Time series imaging, the motivation

As mentioned previously, there is currently no technical difficulty in conducting time series imaging, i.e., video recording. Almost all modern array detectors and cameras, both in our daily lives and in research, have video recording functions that allow the user to easily capture a sequential time series of images with customizable time intervals and exposure times. What is truly challenging, however, is maintaining a high enough imaging speed while fulfilling the requirements of the other aspects of the "frustration pyramid," such as high resolution, 3D imaging, multi-color imaging, and high enough SNR, all of which can necessitate a significant

decrease in frame rate. When conducting high-dimensional super-resolution imaging, the acquisition for a single frame of a data cube could be prodigiously long. Taking multi-color 3D-SIM for example, to image 3 slices and 3 color channels, 9 individual patterns are needed at each z-position and for each color channel for super-resolution SIM image reconstruction, leading to a total of 81 exposures to acquire a single frame image! If no improvements can be made this would necessitate an imaging rate that is which is almost 2 orders of magnitude slower than normal wide field imaging! The slow imaging speed originates from the need to sequentially scan over each different dimension: scanning across different focal planes for 3D imaging, scanning across different color channels for multi-color imaging, and scanning across the pattern series for super-resolution reconstruction.

A natural question to ask is: is it possible to simultaneously image multiple focal planes, multiple color channels, and multiple illumination patterns so that the need for sequential imaging can be eliminated, and a high-dimensional super-resolution image can be captured with only 1 frame? This is the question that I am trying to answer in this dissertation.

1.3 Structure of Dissertation

This Dissertation describes the development of novel snapshot multi-dimensional structured illumination microcopies (SIM) with multiple innovations, including tandem adaptive optics, easily scalable multi-color imaging, multi-plane 3D-SIM, deep-learning empowered virtual multi-channel imaging, and snapshot SIM via compressive sensing. The advances can be used together to achieve a good measure of the optimal improvement in SIM that is desired. Therefore, the primary goal to enable simultaneous imaging of multiple z slices, multiple color, and multiple patterns within a "snapshot" of acquisition is now close at hand.

Chapter 2 presents a theoretical discussion of the principle of SIM and how to use a digital micromirror device (DMD) to generate the patterns required for SIM imaging. The experimental realization of a DMD-based SIM is then presented and demonstrate super-

resolution imaging of fluorescent bead samples and cell samples.

Chapter 3 presents the development of novel tandem aberration correction optics (TACO) system developed to fully correct all kinds of aberrations in SIM (Gong and Scherer (2023)). This system incorporates both conjugate adaptive optics and pupil adaptive optics with distinctively designed algorithms to correct for local aberrations in the illumination path and global aberrations in the detection path separately.

Chapter 4 presents the development of multi-scan SIM system that achieves high speed 3D-SIM imaging by using a multiplane image splitter for simultaneous imaging across multiple planes, complemented by a deformable mirror (DM) for rapid remote focusing and aberration correction (Gong et al. (2024b)).

Chapter 5 presents the development of grating-DMD-SIM system that works as a general solution to achieve scalable multi- color DMD-SIM (Gong et al. (2024a)). A supplementary blazed grating is paired with a DMD to counteract the angular dispersion effect and achieve “automatic alignment” of all the excitation beams.

Chapter 6 presents the application of μ Split-SIM, a convolutional neural network model was adapted and capable of 3 channels demixing for SIM imaging. The novel μ Split-SIM model allows for virtual 3-channel SIM imaging within only 1 exposure time and 1 color label.

Chapter 7 presents Snapshot Compressive Imaging based SIM (SCI-SIM), an image reconstruction algorithm developed to reconstruct a super-resolution image from the single acquisition of an integration of 9 SIM pattern by encoding them with an extra series of SCI patterns.

Chapter 8 presents a comprehensive discussion and outlook of the future of microscopy technique development along the direction of high-dimensional imaging.

Appendix A summarizes other research that I have done focused on the biological problem of intracellular insulin transport in pancreatic β cells (Yi et al. (2024); Gong et al. (2024c)).

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CHAPTER 2

DMD-SIM: DIGITAL MICROMIRROR DEVICE BASED SIM

2.1 Introduction

Compared to other super-resolution techniques, one of the most distinct features of SIM is that it does not require any special labels, such as the on/off switchable fluorescent label for STORM/PALM, replenishable fluorescent labels for STED/RESOLFT, or fluorescently labeled short DNA oligonucleotides for DNA-PAINT. Nor does SIM need any special chemical treatment, such as the gel expansion for ExM. In contrast, SIM is a method that purely bases on the signal processing of the sample images interfering with the illumination pattern. These properties make it widely applicable to almost any imaging condition. In this section I introduce the mathematical theory of SIM to establish a foundation to understand the principle of SIM. I use the perspective of Fourier optics analysis following the original work of SIM (Gustafsson (2000); Gustafsson et al. (2008)), including the cases of 2D imaging, 3D imaging, and some other varieties of SIM.

2.1.1 2D-SIM

For standard 2D-SIM, the illumination is a sinusoidal pattern generated by the interference of 2 coherent illumination beams of light. The resulting intensity distribution of the illumination, $I_{\theta,\phi}(\vec{r})$, can be expressed as:

$$I_{\theta,\phi}(\vec{r}) = I_0 \left[1 - \frac{m}{2} \cos(2\pi \vec{p}_\theta \cdot \vec{r} + \phi) \right], \quad (2.1)$$

where $\vec{r}$ is the space vector in the lateral direction, I_0 is the illumination intensity, $\vec{p}_\theta = [p \cdot \cos(\theta), p \cdot \sin(\theta)]$ is the frequency vector, ϕ is the phase of the sinusoidal pattern, and m is the modulation depth of the interference. For a sample $S(\vec{r})$ imaged in an optical

microscopy system with a point spread function (PSF) $H(\vec{r})$ and illumination pattern $I_{\theta,\phi}(\vec{r})$, the resulting image $D_{\theta,\phi}(\vec{r})$ can be expressed as:

$$D_{\theta,\phi}(\vec{r}) = [S(\vec{r})I_{\theta,\phi}(\vec{r})] \otimes H(\vec{r}) + n, \quad (2.2)$$

where n is a noise term. The Fourier transform of the image $\tilde{D}_{\theta,\phi}(\vec{k})$ can be then expressed as:

$$\tilde{D}_{\theta,\phi}(\vec{k}) = (\tilde{I}_{\theta,\phi}(\vec{k}) \otimes \tilde{S}(\vec{k})) \cdot \tilde{H}(\vec{k}) + \tilde{n}, \quad (2.3)$$

where $\tilde{I}_{\theta,\phi}(\vec{k})$, $\tilde{S}(\vec{k})$, $\tilde{H}(\vec{k})$, and $\tilde{n}$ are the Fourier transform of $I_{\theta,\phi}(\vec{r})$, $S(\vec{r})$, $H(\vec{r})$, and n , respectively. $\vec{k}$ is the frequency space vector. Due to its sinusoidal distribution, the Fourier transform of the illumination pattern have a very simple form:

$$\tilde{I}_{\theta,\phi}(\vec{k}) = I_0(\delta(\vec{k}) - \frac{m}{2} [\delta(\vec{k} - \vec{p}_\theta)e^{-i\phi} + \delta(\vec{k} + \vec{p}_\theta)e^{i\phi}]). \quad (2.4)$$

Therefore, the resulting Fourier transform of the image can be simplified as:

$$\tilde{D}_{\theta,\phi}(\vec{k}) = I_0(\tilde{S}(\vec{k}) - \frac{m}{2} [\tilde{S}(\vec{k} - \vec{p}_\theta)e^{-i\phi} + \tilde{S}(\vec{k} + \vec{p}_\theta)e^{i\phi}]) \cdot \tilde{H}(\vec{k}) + \tilde{n}, \quad (2.5)$$

which is a linear combination of three parts of the frequency information of the sample: $\tilde{S}(\vec{k})$, $\tilde{S}(\vec{k} + \vec{p}_\theta)$, $\tilde{S}(\vec{k} - \vec{p}_\theta)$. The latter 2 terms are supposed to be partially outside the range of spectral frequency information that can be collected by the optical system, thus containing frequency information that allows transcending the diffraction limit. In order to solve these three terms, at least three independent measurements are needed. Typically this is done by using three different phase values of the ϕ of the illumination pattern and as

represented in the following system of linear equations:

$$\begin{bmatrix} \tilde{D}_{\theta,\phi_1}(\vec{k}) \\ \tilde{D}_{\theta,\phi_2}(\vec{k}) \\ \tilde{D}_{\theta,\phi_3}(\vec{k}) \end{bmatrix} = I_0 \begin{bmatrix} 1 & -\frac{m}{2}e^{-i\phi_1} & +\frac{m}{2}e^{-i\phi_1} \\ 1 & -\frac{m}{2}e^{-i\phi_2} & +\frac{m}{2}e^{-i\phi_2} \\ 1 & -\frac{m}{2}e^{-i\phi_3} & +\frac{m}{2}e^{-i\phi_3} \end{bmatrix} \begin{bmatrix} \tilde{S}(\vec{k})\tilde{H}(\vec{k}) \\ \tilde{S}(\vec{k} - \vec{p}_\theta)\tilde{H}(\vec{k}) \\ \tilde{S}(\vec{k} + \vec{p}_\theta)\tilde{H}(\vec{k}) \end{bmatrix} + \begin{bmatrix} \tilde{n}_1 \\ \tilde{n}_2 \\ \tilde{n}_3 \end{bmatrix}. \quad (2.6)$$

The system of linear equation can be solved as:

$$\begin{bmatrix} \tilde{S}(\vec{k})\tilde{H}(\vec{k}) \\ \tilde{S}(\vec{k} - \vec{p}_\theta)\tilde{H}(\vec{k}) \\ \tilde{S}(\vec{k} + \vec{p}_\theta)\tilde{H}(\vec{k}) \end{bmatrix} = M^{-1} \begin{bmatrix} \tilde{D}_{\theta,\phi_1}(\vec{k}) \\ \tilde{D}_{\theta,\phi_2}(\vec{k}) \\ \tilde{D}_{\theta,\phi_3}(\vec{k}) \end{bmatrix}, M = I_0 \begin{bmatrix} 1 & -\frac{m}{2}e^{-i\phi_1} & +\frac{m}{2}e^{-i\phi_1} \\ 1 & -\frac{m}{2}e^{-i\phi_2} & +\frac{m}{2}e^{-i\phi_2} \\ 1 & -\frac{m}{2}e^{-i\phi_3} & +\frac{m}{2}e^{-i\phi_3} \end{bmatrix}, \quad (2.7)$$

The estimation of the super-resolution components $\tilde{S}(\vec{k} + \vec{p}_\theta)$ and $\tilde{S}(\vec{k} - \vec{p}_\theta)$ can then be acquired by employing deconvolution methods such as Wiener filtering or RL deconvolution (Chen et al. (2023)). These spatial frequency components can then be shifted to the corresponding locations centered at $+\vec{p}_\theta$ and $-\vec{p}_\theta$. Combining with the frequency component $\tilde{S}(\vec{k})$, the information of these three components allows for a super-resolution reconstruction along the direction of θ . To acquire a nearly isotropic super-resolution image, one typically uses three different θ s with 60° intervals to make the complete spatial frequency distribution to be nearly a circle. Considering that three measurements with three values of ϕ at each θ are needed to solve the individual components, 9 images ($3 \text{ angles} \times 3 \text{ phases}$) are required for the reconstruction of a single 2D-SIM image.

Note that in a conventional imaging setup, the spatial frequency of the illumination pattern cannot go beyond the resolution limit, i.e., $|\vec{p}_\theta| \leq k_{max}$, where k_{max} is the diffraction limit of the imaging system. Thus the highest spatial frequency that SIM can acquire is $2k_{max}$, i.e., standard SIM can theoretically achieve $2\times$ better resolution compared to conventional wide-field imaging.

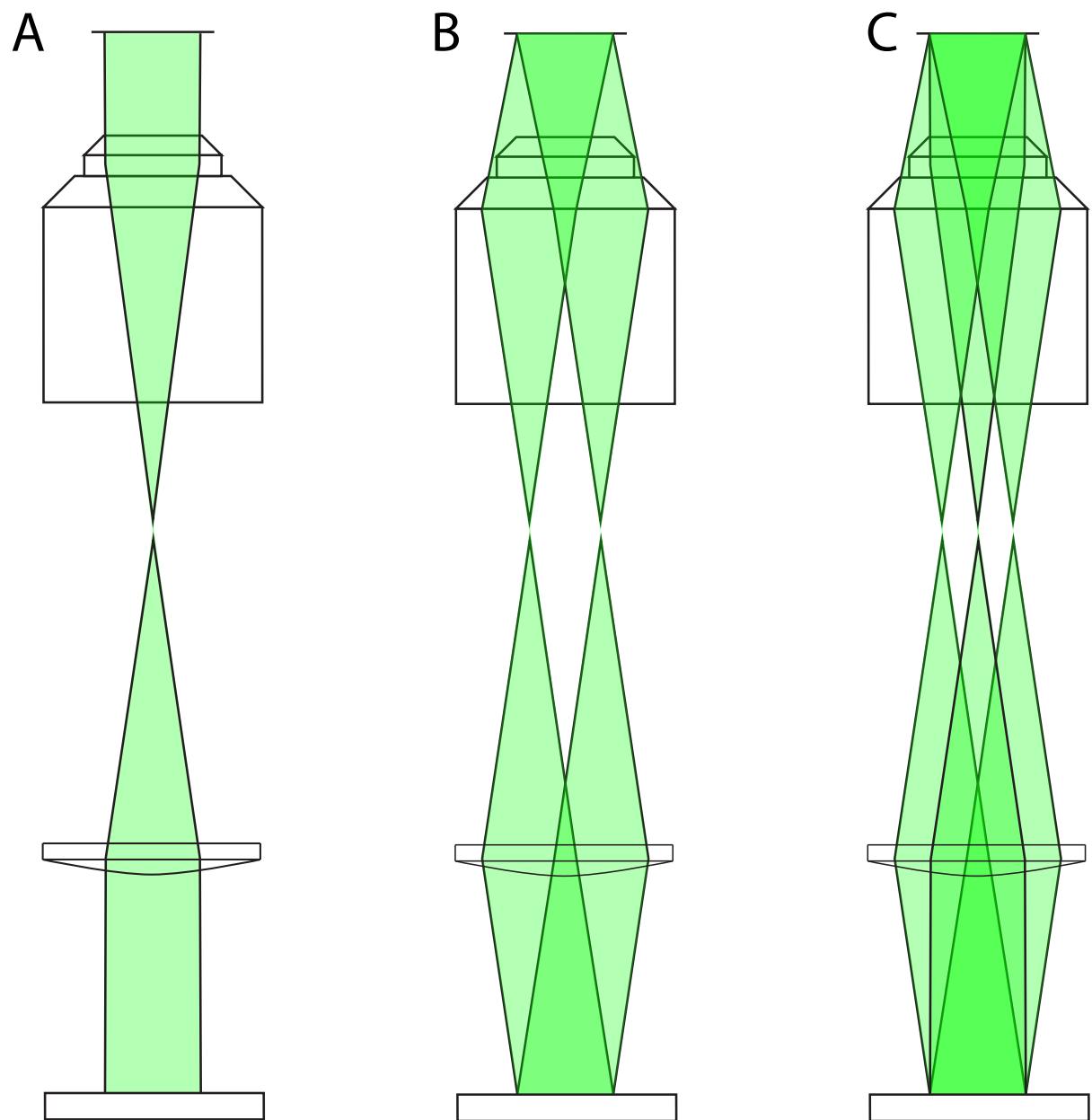


Figure 2.1: **Conventional SIM configuration.** (A) 1-beam-illumination Wide field imaging. (B) 2-beam-interference SIM for 2D imaging. (C) 3-beam-interference for 3D imaging.

2.1.2 3D-SIM

Although 2D-SIM can achieve double the diffraction limited resolution in the lateral direction, it cannot increase the resolution in the axial direction. This is because the pattern generated by 2-beam interference is uniform along the axial direction and does not provide any intensity modulation in the axial direction. This lack of axial resolution enhancement makes 2D-SIM still suffer from issues like out-of-focus light, which is quite common in biological samples. To enhance the resolution in the axial direction, 3-beam interference can be used, which can generate patterned illumination not only in the lateral direction but also in the axial direction. In 3-beam interference, an additional 0-order beam is used to interfere with the ± 1 order beams, and the resulting illumination intensity can be expressed as (Chen et al. (2023)):

$$I_{\theta,\phi}(\vec{r}, z) = I_0 |1 + 2m \cdot e^{i2\pi p_z z} \cos(2\pi \vec{p}_{\theta,r} \cdot \vec{r} + \phi)|^2. \quad (2.8)$$

Equation 2.8 can be expanded as:

$$I_{\theta,\phi}(\vec{r}, z) = I_0 (1 + 2m^2 + 4m \cdot \cos(2\pi p_z z) \cdot \cos(2\pi \vec{p}_{\theta,r} \cdot \vec{r} + \phi) + 2m^2 \cos(4\pi \vec{p}_{\theta,r} \cdot \vec{r} + 2\phi)). \quad (2.9)$$

Assuming a 3D sample $S(\vec{r}, z)$ imaged under a microscopy system with a point spread function (PSF) $H(\vec{r}, z)$ and the illumination pattern $I_{\theta,\phi}(\vec{r}, z)$, the resulting image $D_{\theta,\phi}(\vec{r}, z)$ can be expressed as:

$$D_{\theta,\phi}(\vec{r}, z) = (S(\vec{r}, z) I_{\theta,\phi}(\vec{r}, z)) \otimes H(\vec{r}, z) + n \quad (2.10)$$

The 3D Fourier transform of the image $\tilde{D}_{\theta,\phi}(\vec{k}_r, k_z)$ can be then expressed as:

$$\tilde{D}_{\theta,\phi}(\vec{k}_r, k_z) = (\tilde{I}_{\theta,\phi}(\vec{k}_r, k_z) \otimes \tilde{S}(\vec{k}_r, k_z)) \cdot \tilde{H}(\vec{k}_r, k_z) + \tilde{n}. \quad (2.11)$$

Combining with the sinusoidal form of the illumination pattern, the 3D frequency of the image can be further expressed as:

$$\begin{aligned} \tilde{D}_{\theta,\phi}(\vec{k}_r, k_z) = I_0 & (a_0 S(\vec{k}_r, k_z) + a_1(k_z)(S(\vec{k}_r - \vec{p}_r, k_z)e^{i\phi} + S(\vec{k}_r + \vec{p}_r, k_z)e^{i\phi}) \\ & + a_2(S(\vec{k}_r - 2\vec{p}_r, k_z)e^{i2\phi} + S(\vec{k}_r + 2\vec{p}_r, k_z)e^{i2\phi})) \cdot H(\vec{k}_r, k_z), \end{aligned} \quad (2.12)$$

in which $a_0 = 1 + 2m^2$, $a_2 = 2m^2$, and $a_1(k_z)$ is the Fourier transform of $a_1(z) = 4m \cdot \cos(2\pi p_z z)$ that can be expressed as $a_1 \cdot (\delta(k - p_z) + \delta(k + p_z))$. Similar to 2D-SIM, this shift the high spatial frequency information along the z axis $S(\vec{k}_r \pm \vec{p}_r, k_z - p_z) + S(\vec{k}_r \pm \vec{p}_r, k_z + p_z)$ to be collectible in the objective. To solve all the terms in equation 2.12, 5 individual measurements with 5 different ϕ (i.e., pattern phase shift) values are needed. This imaging information informs following linear equation:

$$\begin{bmatrix} D_{\theta,\phi_1}(k) \\ D_{\theta,\phi_2}(k) \\ D_{\theta,\phi_3}(k) \\ D_{\theta,\phi_4}(k) \\ D_{\theta,\phi_5}(k) \end{bmatrix} = A \begin{bmatrix} S(\vec{k}_r, k_z) \cdot H(\vec{k}_r, k_z) \\ (S(\vec{k}_r - \vec{p}_r, k_z - p_z) + S(\vec{k}_r - \vec{p}_r, k_z + p_z)) \cdot H(\vec{k}_r, k_z) \\ (S(\vec{k}_r + \vec{p}_r, k_z - p_z) + S(\vec{k}_r + \vec{p}_r, k_z + p_z)) \cdot H(\vec{k}_r, k_z) \\ S(\vec{k}_r - 2\vec{p}_r, k_z) \cdot H(\vec{k}_r, k_z) \\ S(\vec{k}_r + 2\vec{p}_r, k_z) \cdot H(\vec{k}_r, k_z) \end{bmatrix}, \quad (2.13)$$

in which

$$A = I_0 \begin{bmatrix} a_0 & a_1 e^{i\phi_1} & a_1 e^{-i\phi_1} & a_2 e^{2i\phi_1} & a_2 e^{-2i\phi_1} \\ a_0 & a_1 e^{i\phi_2} & a_1 e^{-i\phi_2} & a_2 e^{2i\phi_2} & a_2 e^{-2i\phi_2} \\ a_0 & a_1 e^{i\phi_3} & a_1 e^{-i\phi_3} & a_2 e^{2i\phi_3} & a_2 e^{-2i\phi_3} \\ a_0 & a_1 e^{i\phi_4} & a_1 e^{-i\phi_4} & a_2 e^{2i\phi_4} & a_2 e^{-2i\phi_4} \end{bmatrix}, \quad (2.14)$$

The five frequency can be solved as:

$$\begin{bmatrix} S(\vec{k}_r, k_z) \cdot H(\vec{k}_r, k_z) \\ (S(\vec{k}_r - \vec{p}_r, k_z - p_z) + S(\vec{k}_r - \vec{p}_r, k_z + p_z)) \cdot H(\vec{k}_r, k_z) \\ (S(\vec{k}_r + \vec{p}_r, k_z - p_z) + S(\vec{k}_r + \vec{p}_r, k_z + p_z)) \cdot H(\vec{k}_r, k_z) \\ S(\vec{k}_r - 2\vec{p}_r, k_z) \cdot H(\vec{k}_r, k_z) \\ S(\vec{k}_r + 2\vec{p}_r, k_z) \cdot H(\vec{k}_r, k_z) \end{bmatrix} = A^{-1} \begin{bmatrix} D_{\theta, \phi_1}(k) \\ D_{\theta, \phi_2}(k) \\ D_{\theta, \phi_3}(k) \\ D_{\theta, \phi_4}(k) \\ D_{\theta, \phi_5}(k) \end{bmatrix} \quad (2.15)$$

In contrast to 2D-SIM, this 3D-SIM scheme can further shift the high frequency components present in the z direction into the diffraction limited range, thus increasing the axial resolution by up to $2\times$. Typically, a 3D reconstruction will need $3\theta \times 5\phi = 15$ images to reconstruct each axially-resolved SIM image.

2.1.3 Other types of SIM

Apart from the conventional 2D-SIM and 3D-SIM that are based on patterns generated by 2 or 3 beam interference, there have been many other varieties of SIM that utilize different kinds of patterns or techniques. Here, I introduce some typical techniques that have a significant impact in this field.

Multifocal SIM (MSIM) (York et al. (2012, 2013)) uses an array of illumination spots generated by either a digital micromirror device or a physical pinhole array. The pattern generated in this manner does not provide modulation along the z-axis; however, the out-of-focus light can be filtered out by combining the idea of confocal microscopy with the individual pinholes. This method typically takes far more frames (100) to acquire a single slice's super-resolution image since it requires densely scanning the dot array to cover the whole field of view. But the sparsity of this pattern makes image reconstruction relatively simple and can be done in real space, thus the imaging speed can be faster than conventional SIM, and it can even achieve real-time imaging by employing all-analog image processing

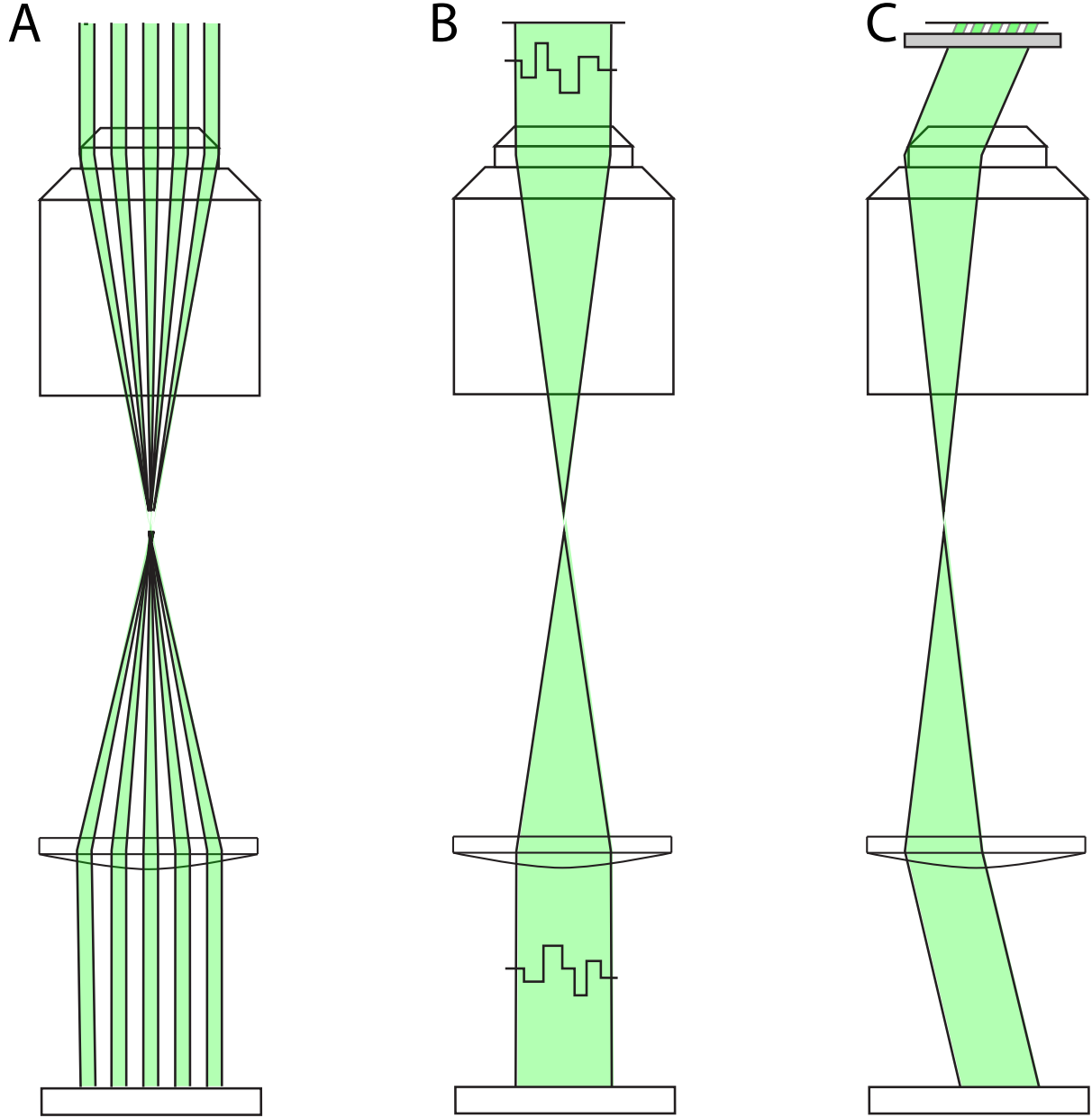


Figure 2.2: **Other types of SIM** (A) Multifocus SIM by using dot array structured illumination. (B) Speckle SIM by using illumination with random speckle pattern. (C) Plasmonic-SIM by using illumination patterns with higher spatial frequency generated by plasmon polaritons.

(York et al. (2013)). Another advantage of reconstruction in real space is that it can be less vulnerable to reconstruction artifacts such as honeycomb or hatching artifacts, which are very common in conventional SIM (Demmerle et al. (2017)).

In contrast to using well-defined patterns, speckle-SIM (Mudry et al. (2012)) utilizes random speckle patterns for super-resolution imaging. In this method, L random patterns are used to illuminate the sample, and L images are acquired. The reconstruction process essentially involves solving for both the sample information S and the L patterns. This can be achieved under the assumption that the illumination can be treated as fully random, i.e., $\sum_1^L \approx LI_0$. Combining this with the L measurements can theoretically solve all the unknown variables. These methods typically require around 150 speckle patterns for super-resolution reconstruction, which can be significantly slower than conventional SIM. However, the biggest advantage of speckle-based SIM is that it does not need any prior knowledge of the illumination pattern for reconstruction. For traditional SIM, successful reconstruction requires very precise determination of the illumination pattern, which can be very challenging, especially when imaging biological samples that are usually dim, disordered, and aberrative. In this situation, the absence of the need to determine the pattern parameter can be of real value. Speckle-SIM is also referred to as blind-SIM because of this feature.

Nonlinear-SIM is another emerging class of SIM-based super-resolution techniques that are attracting more and more attention. While conventional SIM has an upper limit to the resolution it can improve, nonlinear-SIM offers theoretically unlimited resolution by exploiting nonlinear effects. For instance, saturated SIM (Heintzmann and Gustafsson (2009)) uses patterned illumination with very high intensity so that the fluorophores are saturated in some regions. This saturated pattern, distinct from the sinusoidal illumination pattern, will contain higher harmonics that can be used to further extend the spatial frequency that can be collected. Although this method can theoretically work, the requirement for high illumination intensity is usually unrealistic for imaging biological samples.

Another new type of nonlinear SIM is plasmonic-SIM (Wei et al. (2014)). Plasmonic-SIM utilizes surface plasmon polaritons (SPP) that occur when light travels along metallic surfaces. Instead of following the conventional $k = \omega/c$ relationship, the spatial frequency of SPP is determined by the dielectric functions of the metal and can reach far higher k values that can be used for SIM imaging. Due to the complexity of its realization, plasmonic-SIM has not been widely applied currently, but its potential for achieving unlimited resolution with no need for high illumination intensity is very promising.

2.2 Digital micromirror device based SIM

The key step in conducting SIM is the generation of illumination patterns with variable orientations and phases. Initially, this was achieved by physically moving a diffraction grating (Gustafsson (2000)). However, this solution can be very slow, and the instability of the mechanical movement can prevent robustness in imaging. Spatial light modulators (SLMs) have since been widely used for SIM pattern generation due to their relatively faster frame rate and ease of control (O’Holleran and Shaw (2012); Zhao et al. (2021)). Another straightforward method for generating interference patterns is a combination of 6 single-mode fibers, which allows for convenient intensity or polarization control of individual beams (Ortkrass et al. (2023)).

In my work, the illumination pattern is generated using a digital micromirror device (DMD), which has become increasingly attractive in recent years (Chakrova et al. (2015)). A DMD is composed of an array of a large number of micromirrors, where each mirror can be independently switched in a binary fashion between on/off states. This array of micromirrors essentially function as a binary display with micromirrors as its pixels. Compared to other pattern generation solutions, using a DMD offers the following advantages:

- the unparalleled updating speed. Typically, a DMD can have a refresh rate larger than 10 kHz, whereas a liquid crystal-based SLM only has around 100 Hz. This high

speed of a DMD makes it more suitable for high-speed SIM imaging, which is becoming increasingly important for modern bioimaging. Furthermore, the high speed allows for exploring more possibilities of SIM imaging. For instance, in the SCI-SIM Chapter 7, I am going to discuss the possibility of imaging 9 different patterns required for SIM reconstruction with only 1 integrated exposure. Achieving this to study dynamic process will require a very fast pattern update rate (>100 fps).

- the flexibility of displaying different patterns. As mentioned, a DMD is essentially a display and it can display more than just striped patterns. For instance, York et al. (2012) uses a DMD to generate a dot array pattern to achieve MSIM. He et al. (2022) uses an extra DMD to display random binary patterns to combine compressive sensing with SIM imaging. In contrast, the optical fiber strategy can only be used to generate sinusoidal interference patterns. Although SLMs can have complicated modulation to the wavefront of the illumination light, they are usually pure phase modulation and it requires at least 2 SLMs to arbitrarily manipulate the intensity distribution of the illumination (?).

For the reasons stated above, I believe using a DMD for SIM pattern generation has the biggest potential to push the boundaries of SIM imaging. Therefore, I built a DMD-SIM imaging system is built to conduct traditional SIM imaging. This DMD-SIM setup forms the basis of all the imaging setups introduced in the later chapters of this thesis. In this section, we are going to discuss how this DMD-based SIM works. Note that since this DMD-SIM setup only serves as a basic framework, here I focus more on the general rationale of the design of the imaging setup and imaging procedures. I will discuss more about the quantitative analysis for different imaging tasks in the later chapters.

2.2.1 Diffraction effect of DMD

Although DMDs are classified as a kind of amplitude spatial light modulator, we need to notice that the lattice structure of the micro-mirror array also makes DMD a diffraction grating (Fig. 2.3). When using incoherent light sources, this will not be a problem due to the lack of spatial coherence. However, if using coherent light sources, the grating will give rise to a complicated diffraction pattern that is related to the incident angle and wavelength. Thus, a careful analysis of the diffraction characteristics is needed to figure out an optimal illumination configuration. Here, we use numerical Fourier optics analysis to analyze the diffraction effect of the DMD. In the gDMD-SIM chapter, we will also show an analytical approach based on solving the diffraction equations to analyze the DMD.

The DMD can be treated as a transmission optical element that is described by a transmittance function t . For a uniform light field propagating through the device at normal incidence, it will be modulated by t in both phase and amplitude, and the effect should be equivalent to the output of a DMD when illuminated by incident light at a certain angle. The corresponding diffraction pattern in the spectral domain will then be the intensity of the Fourier transform of the transmittance function t .

Consider a DMD with an $(2M+1) \times (2N+1)$ micro-mirror array with each micro-mirror labeled by (m, n) , where $m = -N, -N-1, \dots, N$, $n = -N, -N-1, \dots, N$. Then the aperture of a single micro-mirror can be expressed as

$$\begin{aligned}
 t_0(m, n) &= \text{rect} \left(\frac{x - (n-m)\frac{\sqrt{2}}{2}\delta_0}{\delta_1 \cos \psi} + \frac{y - (m+n)\frac{\sqrt{2}}{2}\delta_0}{\delta_1} \right) \\
 &\quad \cdot \text{rect} \left(\frac{x - (n-m)\frac{\sqrt{2}}{2}\delta_0}{\delta_1 \cos \psi} - \frac{y - (m+n)\frac{\sqrt{2}}{2}\delta_0}{\delta_1} \right) \\
 &= \text{rect} \left(\frac{x_{mn}}{\delta_1 \cos \psi} + \frac{y_{mn}}{\delta_1} \right) \cdot \text{rect} \left(\frac{x_{mn}}{\delta_1 \cos \psi} - \frac{y_{mn}}{\delta_1} \right)
 \end{aligned} \tag{2.16}$$

Where $x_{mn} = x - (n-m)\frac{\sqrt{2}}{2}\delta_0$, $y_{mn} = y - (m+n)\frac{\sqrt{2}}{2}\delta_0$ are the coordinate relative to the

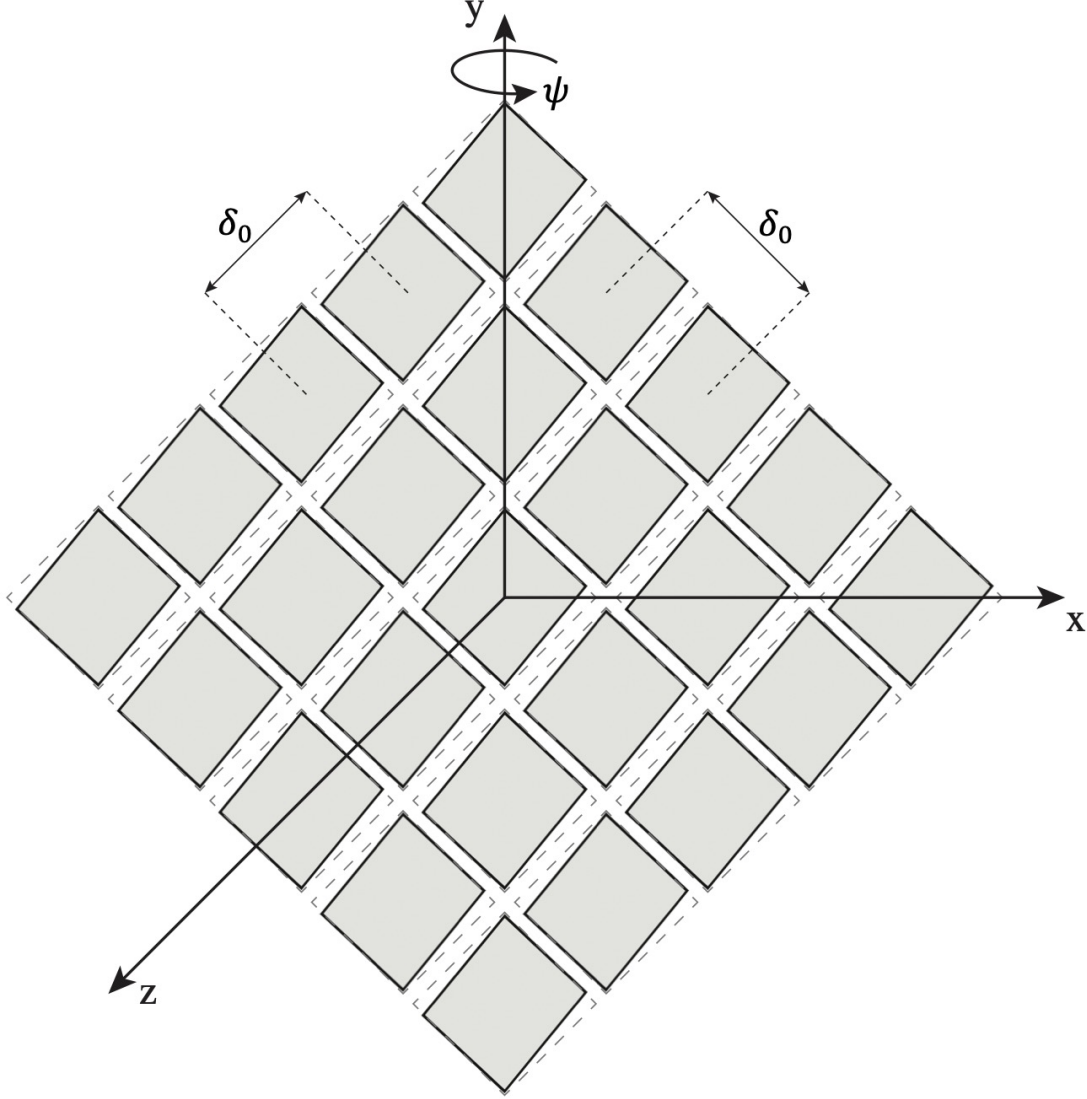


Figure 2.3: **Schematics drawing of DMD's structure.** δ_0 is the interval between adjacent micromirrors. ψ is the tilt angle of the micromirrors. Since the micromirrors are tilting along their diagonals, coordinate system is set with the y axis align with the diagonal for the convenience of equations expression.

$(m, n)^{th}$ micro-mirror, rect is rectangular function.

Here we introduce δ_1 which stands for the actual length of a single micro-mirror as shown in Figure 2.3. The DMD we used has a fill factor of 92%, which makes $\delta_1 = \sqrt{0.92}\delta_0$. The tilting angle ψ of micro-mirror contributes a phase modulation term and t_0 can be rewritten as:

$$t_0(m, n) = \text{rect}\left(\frac{x_{mn}}{\delta_1 \cos \psi} + \frac{y_{mn}}{\delta_1}\right) \cdot \text{rect}\left(\frac{x_{mn}}{\delta_1 \cos \psi} - \frac{y_{mn}}{\delta_1}\right) \cdot \exp\left[\frac{i2\pi x_{mn}}{\lambda} \left(\frac{\sin(\theta_0 - \psi)}{\cos \psi} - \tan \psi\right)\right] \quad (2.17)$$

The obliqueness of the incident ray compared to the surface of DMD (the x-y plane) also contributes an additional phase shift. Therefore, t_0 should be further rewritten as:

$$t_0(m, n) = \text{rect}\left(\frac{x_{mn}}{\delta_1 \cos \psi} + \frac{y_{mn}}{\delta_1}\right) \cdot \text{rect}\left(\frac{x_{mn}}{\delta_1 \cos \psi} - \frac{y_{mn}}{\delta_1}\right) \cdot \exp\left[\frac{i2\pi x_{mn}}{\lambda} \left(\frac{\sin(\theta_0 - \psi)}{\cos \psi} - \tan \psi\right)\right] \cdot \exp\left[\frac{i2\pi}{\lambda} (n - m) \frac{\sqrt{2}}{2} \delta_0 \sin \theta_0\right] \quad (2.18)$$

The whole transmittance function of the DMD can then be expressed as:

$$t = \sum_{m, n} t_0(m, n) \quad (2.19)$$

Under the far-field approximation (Fraunhofer diffraction), the diffraction pattern, T , can be obtained from the Fourier transform of the transmittance function:

$$T(x', y') = \left| \mathcal{F} \left[t\left(\frac{2\pi}{\lambda z}(x, y)\right) \right] \right|^2 \quad (2.20)$$

In the simulation I set $M = N = 25$, $\theta_0 = 24^\circ$, $\lambda = 532$ nm, and obtained the transmittance function shown in Fig. 2.4. Note that only the real part of the 21x21 pixels of the function is shown. I also chose θ_0 to be twice the tilted angle of the micromirrors. This choice of θ_0 is special; the phase shift contribution from individual tilted micromirrors was

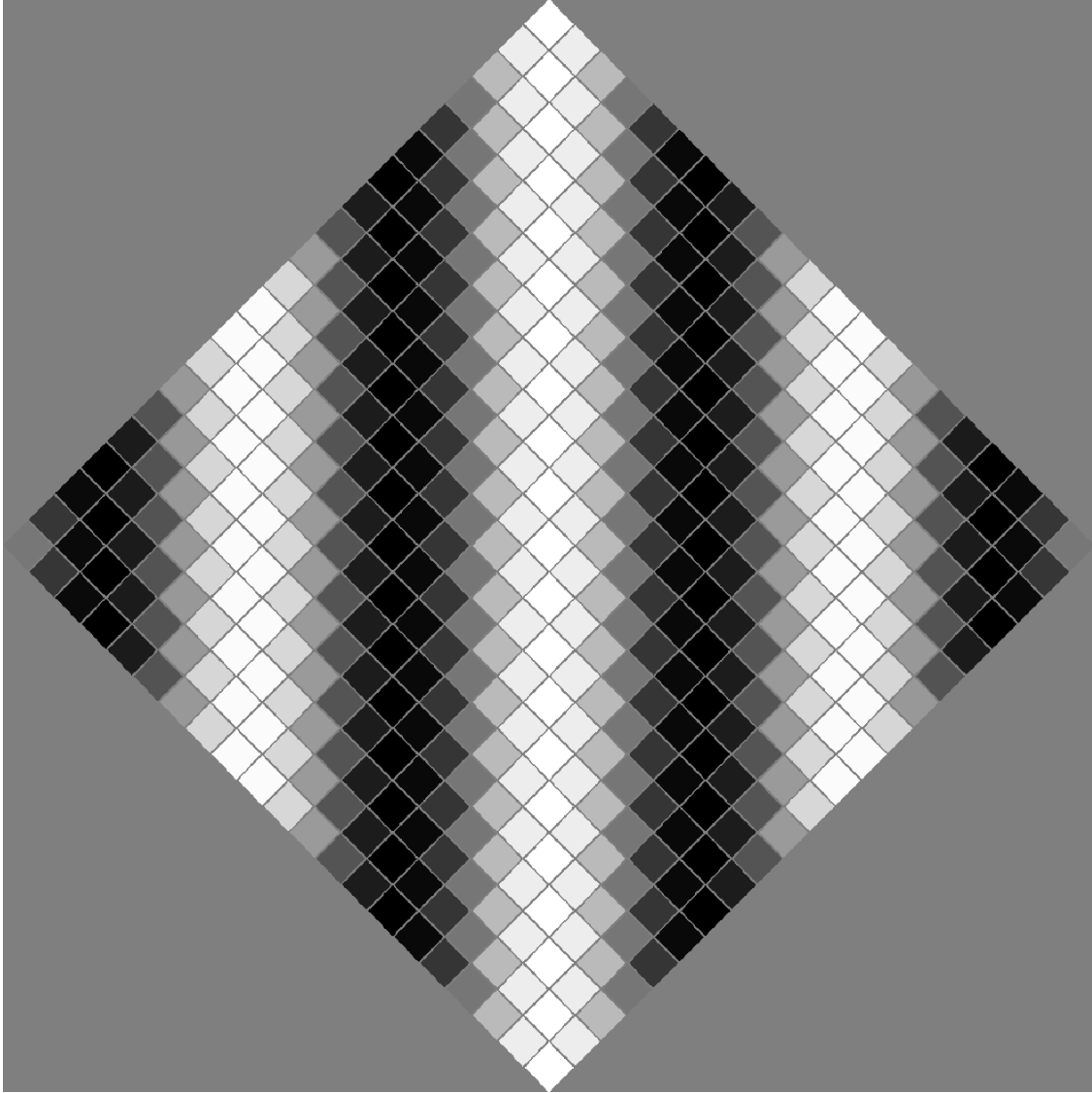


Figure 2.4: **Numerical simulation of the transmittance function of DMD.** The transmittance function is calculated based on equations 2.18 and 2.19

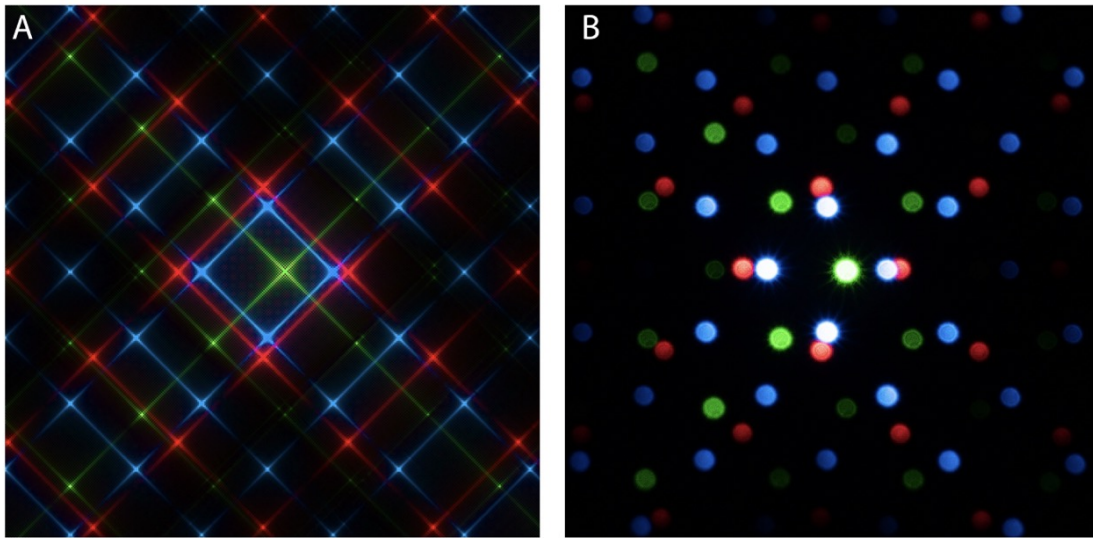


Figure 2.5: **Numerical simulation and experimental results of DMD diffraction** (A) Simulation of DMD diffraction pattern. (B) Actual photo of the DMD diffraction pattern created in the lab. Note the general correspondance of the simulated and measured patterns. However, the detailed color-dependent diffraction effects in the simulated result are not apparent in the measured image.

actually zero. We also set λ to be 488 nm or 632 nm in other simulations to consider the diffraction effects at two other common laser wavelengths. The simulated results for all three wavelengths are shown collectively in Fig. 2.5 (A). It can be seen that for the 532 nm laser wavelength simulation, there is a bright diffraction order close to the center. While for the 488 nm and 632 nm lasers, the power is distributed to multiple off-center diffraction orders. We further imaged the diffraction pattern of the three lasers, and it shows a perfect match with the simulation results. Note that there is an additional cross-shaped diffraction pattern in the simulation result, which is due to the finite pixel size in the numerical simulation.

From this Fourier optics simulation result, we see that 532 nm lasers can achieve a near-optimal illumination condition in which most of the light intensity is concentrated in a diffraction order close to the center. While for 488 nm and 632 nm, it becomes challenging to achieve efficient illumination. We have to either change the incident angle to use one of the 4 nearest diffraction orders and waste most of the excitation energy, or try to collect the 4 nearest diffraction orders at the same time, which requires a big enough numerical aperture objective and very careful alignment.

In this chapter, I mostly restrict my discussion to the use of the 532 nm laser. However, we will also introduce a new way to do multi-color imaging with a DMD-based SIM system in Chapter 5.

2.2.2 Hardware setup

Figures 2.6 and 2.7 illustrate 2D and 3D representations of a standard DMD-SIM imaging setup, respectively. All the imaging setups in the later chapters of this thesis are based on this setup with various modifications. Detailed descriptions of the setup will be provided in those chapters. Therefore, instead of just listing all the optics parts and parameters, I would like to focus more on the rationale behind building the light path.

Firstly, a 532 nm laser is used as the light source. As discussed in the previous subsection,

532 nm can achieve near-blazed grating condition with the output light perpendicular to the DMD interface, which can be very convenient for optical aligning and also has much better light efficiency. While for other common wavelengths such as 488 nm or 642 nm, the blazed grating condition can only be fulfilled with very arbitrary output angles, which makes the alignment much more difficult. For this reason, 532 nm is chosen to be the main light source for most of the imaging tasks. In Chapter 5, we will discuss how to implement other laser wavelengths to conduct multi-color imaging.

Another practical reason for choosing the 532 nm laser is that compared to other diode lasers, the Spectra-Physics Millennia V laser in our lab has the highest output power, which could be very useful not only for imaging but also for aligning and calibration. The lowest output power of the 532 nm laser is 200 mW, which is still pretty high for most bioimaging applications, so an extra module for intensity adjustment is needed. Here, a combination of a pair of polarizers and a half-waveplate is used to conveniently adjust the illumination power. The reason for not using a simple neutral density (ND) filter is because it is based on light absorption and thus will cause spatially-dependent changes in the index of refraction of the ND filter, concomitant lensing of the transmitted beam, and even has the danger of being burned by the laser. For the same reasons, polarizer 2 cannot be a basic film polarizer; Instead, a Glan-Taylor type of polarizer is used to reflect the light with incorrect linear polarization to its side port instead of absorbing the light, thus achieving safe intensity adjustment with high power input light. Apart from intensity adjustment, this module can also adjust the polarization of the transmitted light to the rest of the optical setup. This is crucial when working with an SLM as we will see in the Chapter 3.

After the polarizers and halfwave plate, a pair of lenses is used to expand the laser beam. Here, an expansion factor of $7.5\times$ is used in order to cover the whole DMD array, which has dimensions of roughly 12×20 mm. Although a $7.5\times$ beam expansion can cover the DMD, it sacrifices illumination intensity, which will be crucial for other lasers that do not have as

high output power or diffraction efficiency. For this reason, in most other works, I swap the 750 mm lens with a 400 mm lens to decrease the beam expansion to $4\times$. This changes the illumination spot from a circumscribed circle to an inscribed circle of the DMD chip. In most cases, it is big enough for wide-field-based imaging and can save a lot of laser power for sample illumination.

After the 100 mm lens, a diamond pinhole aperture is used to clean up the beam profile. The aperture can effectively filter out some extra random and higher-order modes of the laser beam. However, it also reduces the illumination power. In the gDMD-SIM chapter, we removed this aperture since it cuts off too much power of other lasers and their unfiltered spatial modes were good.

After beam profile expanding and cleaning, the beam hits the DMD at an incident angle of 24° . As discussed in the previous section, this can achieve the blaze condition, and the primary output beam is perpendicular to the DMD. Due to beam relaying, this makes the DMD pattern be in a single focal plane in the sample. For SIM imaging, a series of binary patterns is displayed to act as a binary grating to generate diffraction beams.

After the DMD, only the ± 1 and 0 order diffraction beams are collected to achieve 3-beam interference. Instead of directly sending the light to the tube lens and objective, an extra pair of lenses is used after the DMD. This additional lens pair relays the pupil plane and the image plane, which usually lie inside the microscope body, to the free space region outside, making it convenient to manipulate. For instance, a polarizer can be placed at the pupil plane to optimize the polarization of the incident light to achieve optimal interference modulation. In the Chapter 3, an aberration slide is also placed at the sample plane in the relay lens system to simulate the case of strong aberration. This pair of lenses can also be used to adjust the conjugate relationship between the DMD surface and the sample plane, which is frequently used during alignment. The choices of the focal length of the relay lenses can be varied flexibly. Therefore, in combination with the choices of DMD pattern, different

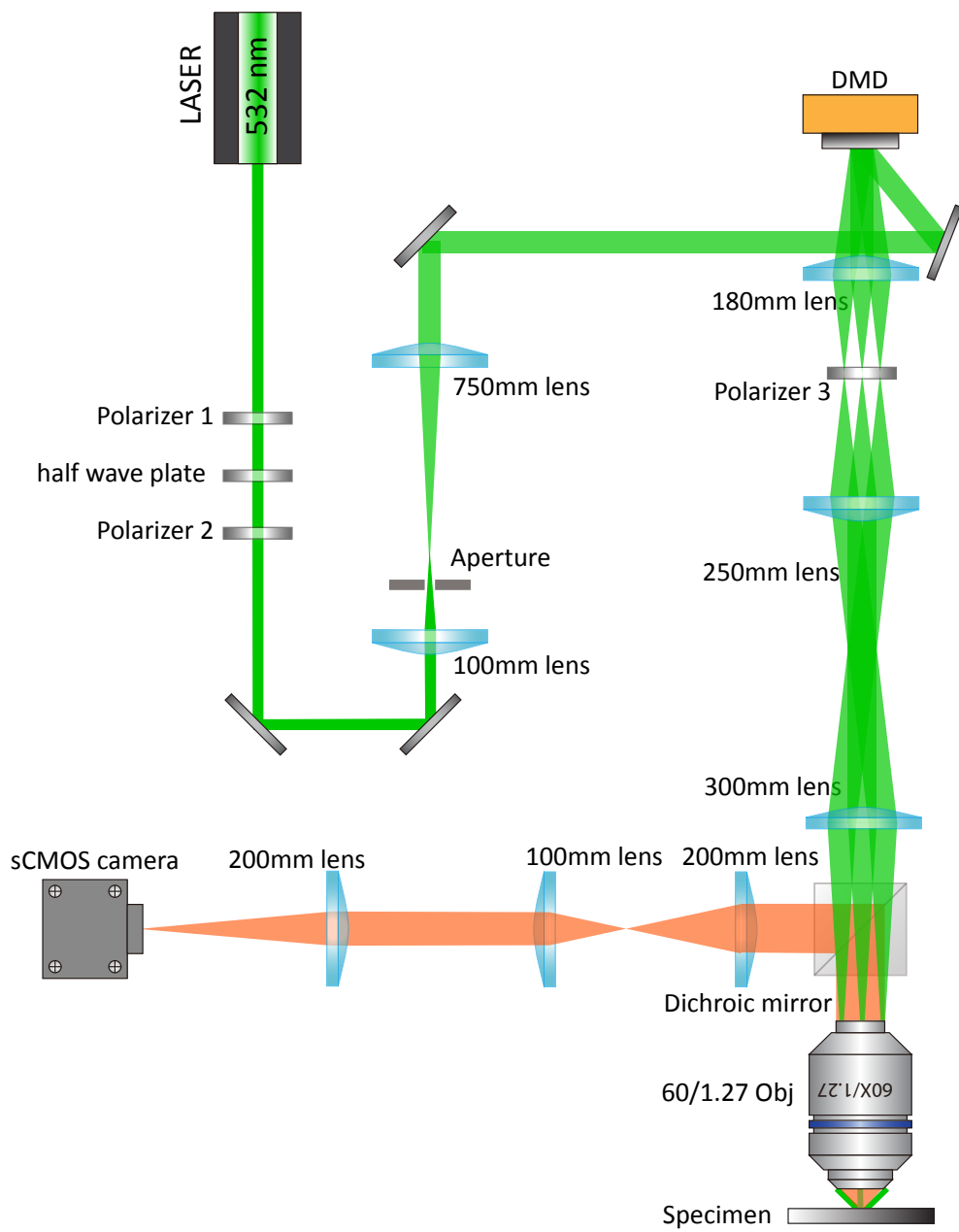


Figure 2.6: **2D schematic of the DMD-SIM setup.** The detailed description of the light path is presented in the main text.

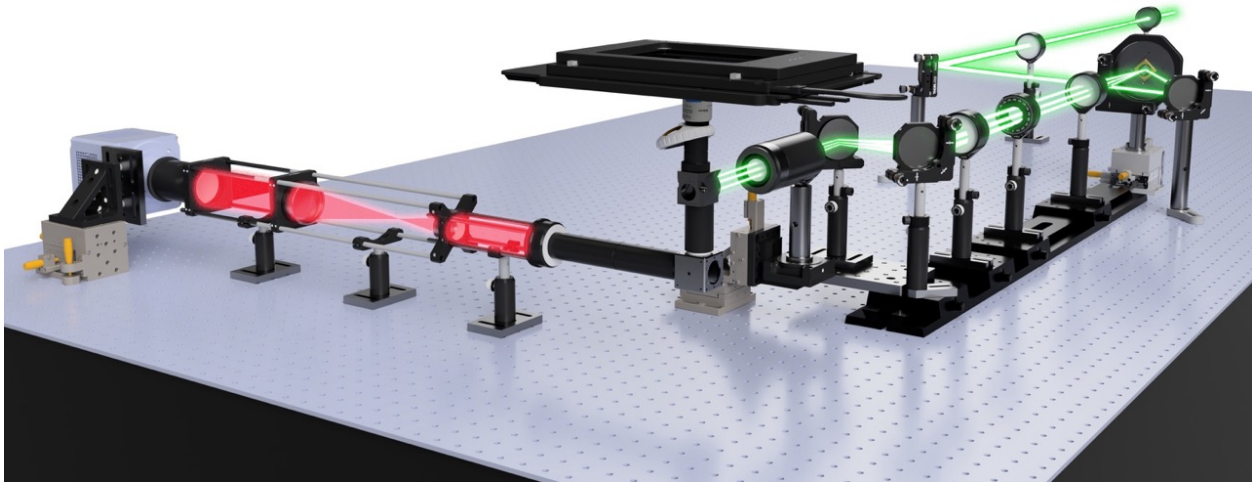


Figure 2.7: **3D schematic of the DMD-SIM setup.** Colors show the optical beam for illumination (green) and emission (red).

pattern frequencies at the sample plane can be achieved.

For my work, multiple combinations have been tested, and a 180 mm/250 mm lens pair and a 6-pixel DMD pattern are determined to have the best performance when combined with a 300 mm tube lens and a $60\times$ 1.27 NA objective. Note that here the tube lens is actually a 300 mm focal length doublet lens, instead of a standard 200 mm lens. This is because the physical distance between the tube lens port of the microscope body and the objective is actually more than 300 mm. For conventional point-scanning microscopes that utilize an infinite focus system, the distance between the tube lens and objective does not really matter. However, SIM is a wide-field based imaging system, and it is desirable to make the tube lens and the objective form a 4F system. For this reason, a 300 mm lens is used as the tube lens.

In the detection path, instead of directly mounting the sCMOS array detector to the microscope body, an extra pair of lenses is used to provide a $2\times$ expansion. One of the main reasons for this is that the sCMOS detector used in this system is the Photometrics

Kinetix, which has a sensor with a diagonal length (dimension) of 30 mm, which is much larger compared to conventional c-mount cameras. Therefore, an extra $2\times$ magnification is used to cover the whole size of the array detector.

More importantly, similar to the extra lens pair in the illumination path, this additional lens pair in the detection path also extends the pupil plane and image plane, providing room for future extensions. For instance, in the TACO chapter, a deformable mirror is placed at the pupil plane to conduct aberration correction. In the MS-SIM chapter, an image-plane-splitter is placed at the image plane to split the fluorescence light into three individual beam paths.

As can be seen, this standard DMD-based SIM setup is quite simple and straightforward, yet it serves as a standard framework that contains numerous interfaces and can be extended with multifaceted functions.

2.2.3 *Hardware control*

For the standard SIM setup, three main components need to be actively controlled: the laser, the detector, and the DMD. In our setup, these correspond to the 532 nm laser, the sCMOS array detector, and the DMD, respectively. The schematic of the control relationship of our setup is shown in Figure 2.8.

The laser is simply controlled by a simple optical shutter (Thorlabs, SH05R). It does not need to be strictly synchronized with the other devices because the DMD itself acts like a shutter that passively synchronizes the laser: when the system is not imaging, all the micromirrors of the DMD are in the off state and no laser light can be directed to the sample. Only when the system is actively imaging and the DMD is displaying patterns can the laser light be directed to the sample. This passive shutter function of the DMD ensures that the laser illumination is strictly synchronized with the image acquisition condition of the system, and the other optical shutter is only needed when testing the imaging system.

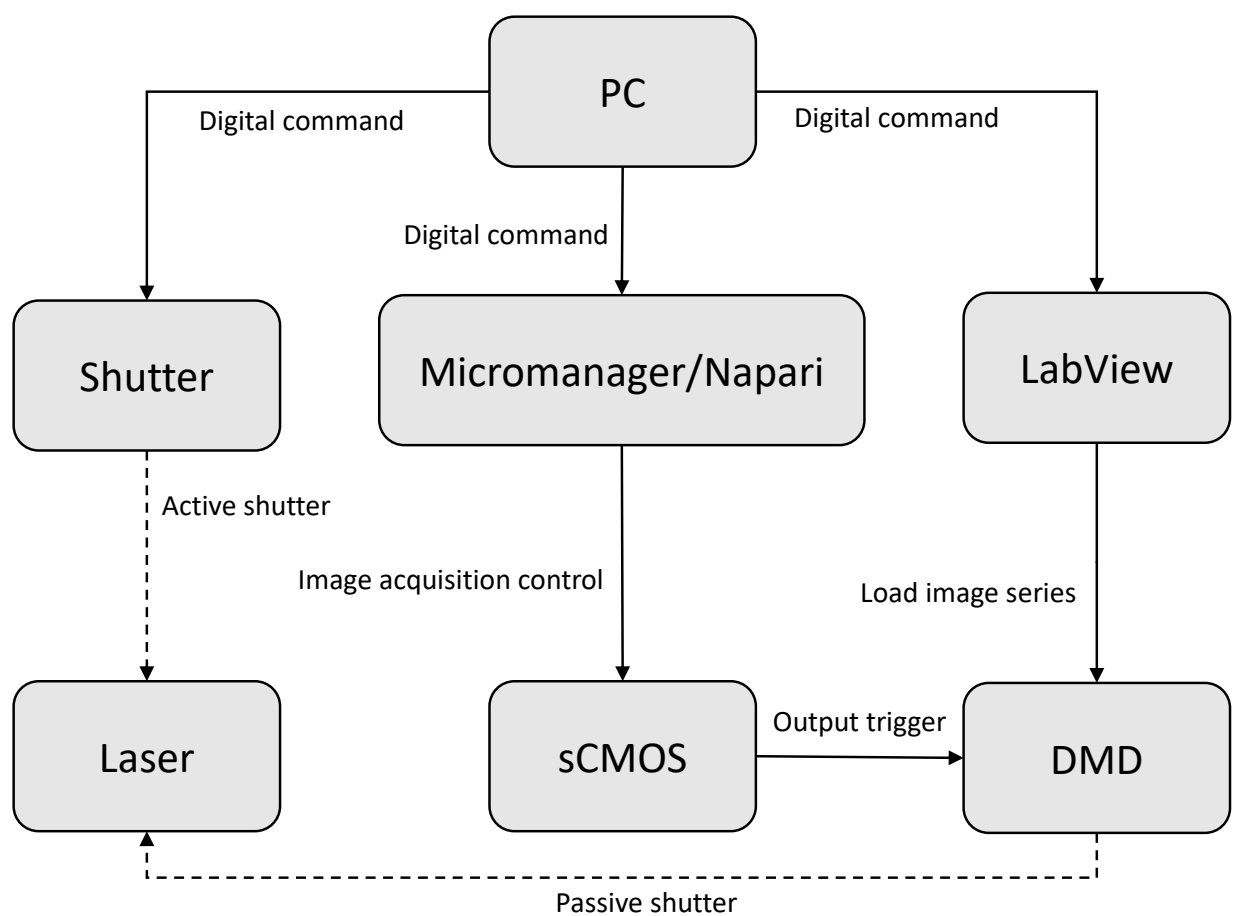


Figure 2.8: **Electronic control of the DMD-SIM.** A detailed description of the electronic control is presented in the maintext.

The sCMOS detector has been controlled through two different platforms: Micromanager or Napari. Both platforms use the Micromanager core and are straightforward to implement. An official Micromanager configuration file is provided by the manufacturer to allow for straightforward control of the detector. Here, we use the array detector as the master device to send trigger-out signals, and the DMD is used in slave mode to receive the signal and synchronize with the sCMOS detector.

For SIM imaging, a series of N binary pattern images is loaded to the DMD, and a time series of N images is acquired. During each image frame acquisition, the DMD displays one pattern, and when imaging the next frame, the DMD switches to the next pattern. A customized LabView program controls the DMD to achieve the required function (see Figure 2.9). The program can be set to four different modes: SIM, SIMCheck, WF, OS. The SIM mode is the standard mode for SIM imaging. In this mode, the DMD is in slave mode and updates its pattern following the signal sent by the camera. In the SIMCheck mode, the DMD constantly displays the SIM pattern to allow for checking the SIM pattern quality. In the WF mode, all the micromirrors are in the "on" state to achieve uniform wide-field illumination without a structured pattern. The OS mode is one special case of SIM imaging where only three patterned images are needed to achieve optical-sectioning imaging without lateral resolution enhancement. The detailed LabView code for the DMD control is provided in Figure 2.14. With this entire controlling setup, automatic SIM imaging acquisition can be achieved robustly.

2.2.4 SIM reconstruction

The image reconstruction is crucial for successful SIM imaging. Although the principle of SIM reconstruction is straightforward, the actual implementation can be very challenging due to issues such as the inaccuracy of pattern parameter determination, low signal-to-noise ratio, and imprecise PSF information. All these issues can lead to severe reconstruction

Figure 2.9: **The front pannel of DMD control software.** Picture time: displaying time of each single image for SIM mode. This value usually should be less or equal than the exposure time. Angle: angle of the pattern used for the SIM check mode. Seq repeat: repeat time of the pattern set. This value usually equals the total number of SIM images. Initialize: initialize the DMD after uploading all the needed pattern image. Restart: restart the device to display the patterns again. Stop: stop the program and release the device.

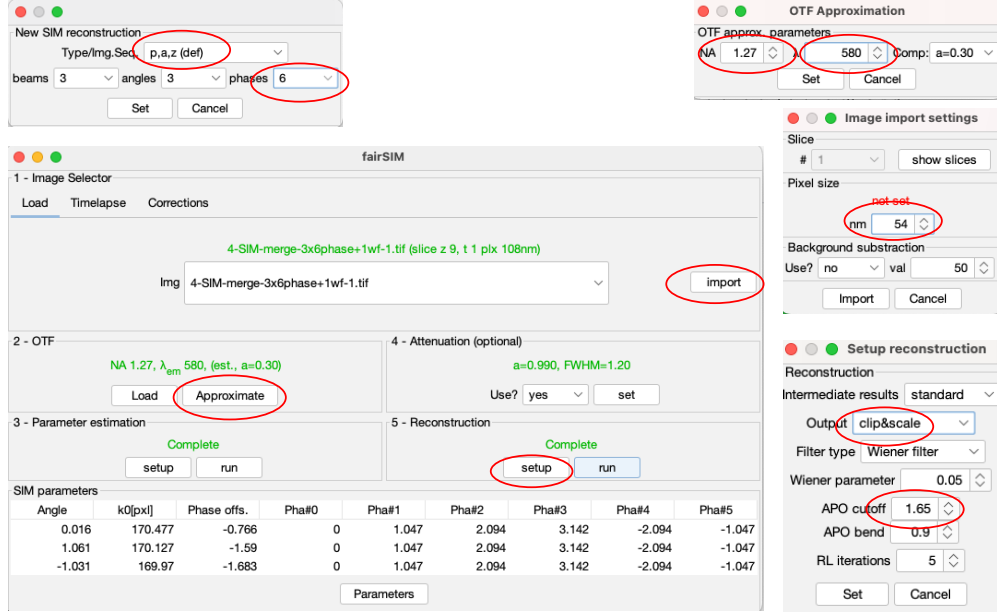


Figure 2.10: **SIM reconstruction by FairSIM.** The red circles mark the parameters that are set to be different from the default setting in order to optimize the reconstruction quality.

artifacts or even failure in image reconstruction (Demmerle et al. (2017)).

Over the past 20 years, many open-source SIM reconstruction software packages have become available (Chen et al. (2023)). However, most of these software packages are specifically designed for certain imaging conditions and cannot provide general usability. Among all the available software, three have been proven to be functional for most imaging situations and have been widely adopted: OpenSIM (Lal et al. (2016); Cao et al. (2023)), HiFiSIM (Wen et al. (2021)), and FairSIM (Müller et al. (2016)). All three software packages can achieve robust image reconstruction with suppressed artifacts. OpenSIM and HiFi-SIM are MatLab-based, while FairSIM is a Java-based ImageJ plugin. For most of the image reconstruction presented here, FairSIM is used for reconstruction due to its better compatibility with MacOS. However, in the Chapter 4, the OpenSIM software is used for 3D reconstruction. The common setup for FairSIM imaging reconstruction is shown in Figure 2.10.

2.3 Experimental result

In this section, we present some typical examples of SIM imaging on fluorescent bead samples and biological cell samples. These results are intended to serve as qualitative demonstrations of SIM imaging. Quantitative analysis of the SIM imaging performance will be presented in the later chapters.

2.3.1 *Generation of SIM patterns*

The SIM patterns can be observed by projecting the SIM illumination onto a near-uniform single-layer sample, such as a dense layer of fluorescent beads. As shown in Figure 2.11, the fluorescent beads form a nearly uniform layer that allows for clear observation of the illumination pattern. It can be seen that the pattern generated by the DMD is very well defined. The pattern period is measured to be around 700 nm, which corresponds to the period of 6 DMD pixels. Note that this is the spatial frequency of the first harmonic, and the second harmonic will have a spatial frequency of around 350 nm.

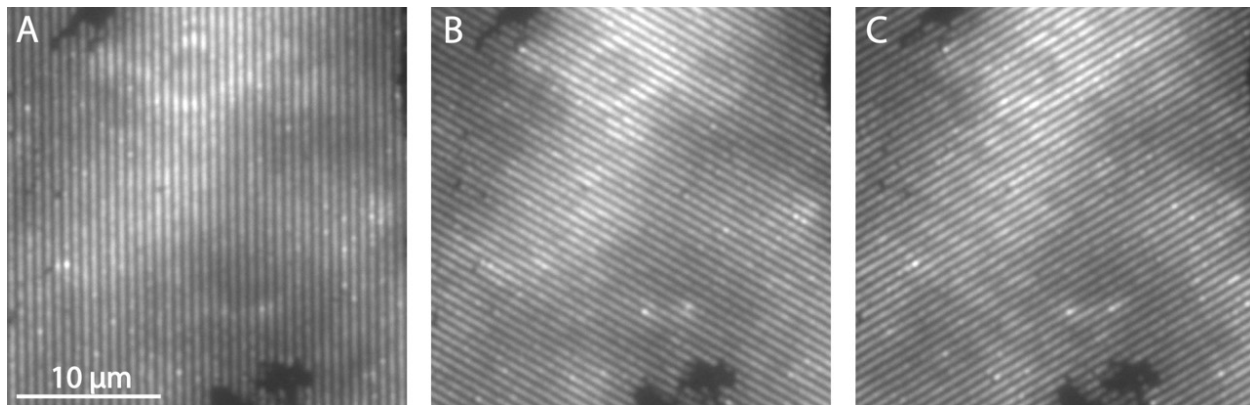


Figure 2.11: **SIM pattern on uniform fluorescent beads layer.** Note the striped illumination pattern at different angles; (A) 0 degree, (B) -60 degree, (C) 60 degree. Each image will be phase shifted in the direction perpendicular to the long axis of the stripes.

2.3.2 SIM imaging of beads

After confirming that the imaging system is capable of projecting well defined illumination patterns onto the sample plane, SIM imaging can be tested on a simple fluorescent bead sample. Figure 2.12 shows an example of wide-field (WF) and SIM imaging of fluorescent beads. After SIM reconstruction, the image shows a significant improvement in resolution. As shown in Figure 2.12C & D, the beads that are originally indistinguishable in WF imaging can then be well distinguished in SIM imaging.

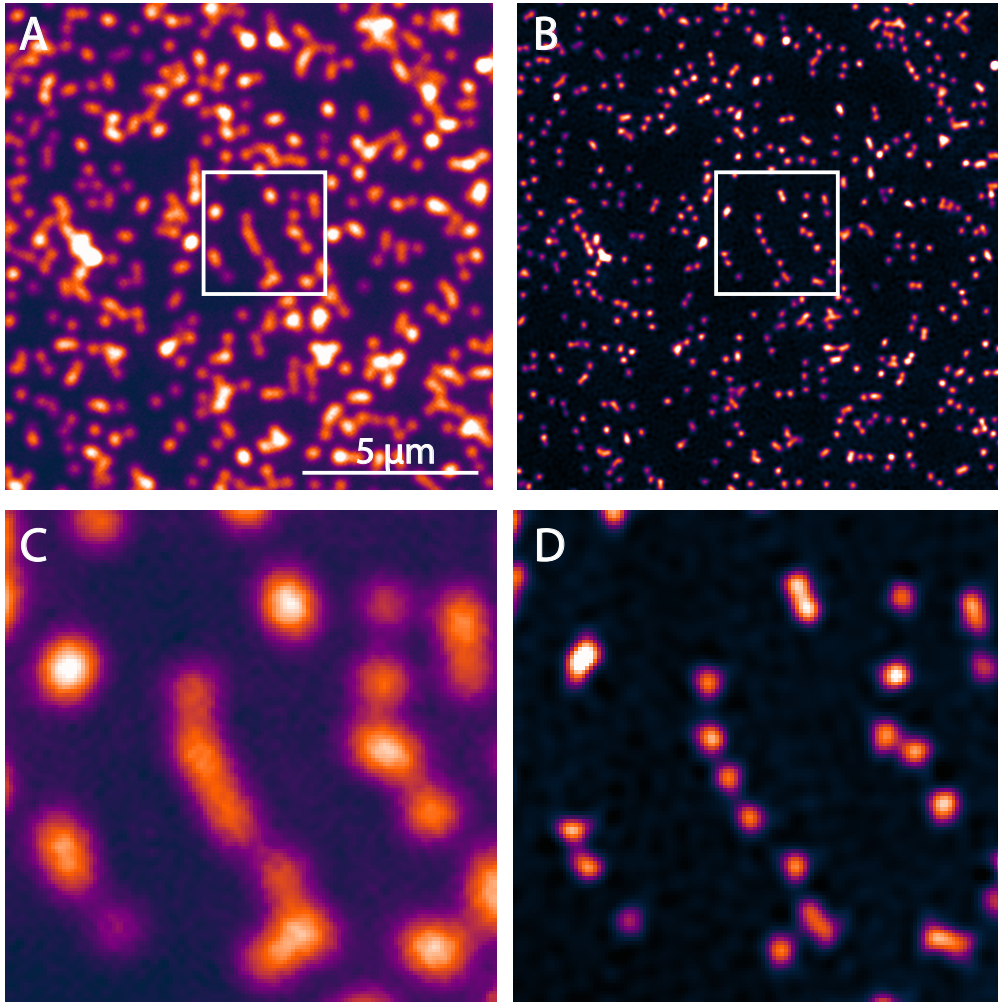


Figure 2.12: **SIM imaging of fluorescent beads sample.** (A) Wide field image of fluorescent beads. (B) SIM image of fluorescent beads. (C,D) Zoomed in images of the framed areas in A and B. The resolution enhancement is near to $2\times$. More precise determinations of the resolution enhancement are presented in Chapter 3 and 5.

2.3.3 SIM imaging of live cells

In contrast to a 2D fluorescent bead sample, a fluorescent-labeled cell sample possesses a 3D distribution of intensity and complicated dynamics along the time dimension, thus making it much more challenging to image. Here, we tested SIM imaging on cell actin networks in live cells. Figure 2.13 shows a significant increase in resolution empowering SIM to wide-field microscopy. Furthermore, since the 3-beam interference allows for optical sectioning, the SIM image can filter out much of the out-of-focus light and shows a much cleaner background compared to wide-field imaging.

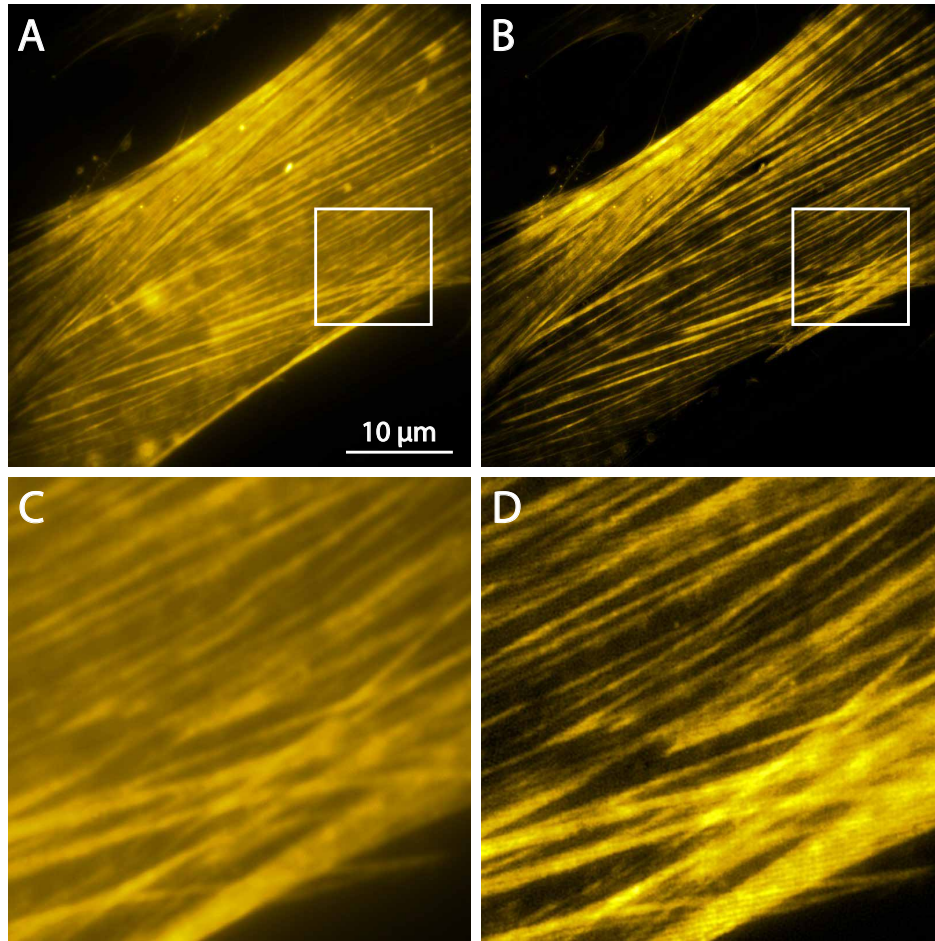


Figure 2.13: **SIM imaging of the fluorescent actin network in live fibroblast cells.** (A) Wide field image of actin. (B) SIM image of actin. (C,D) Zoomed in images of the framed areas in A and B. Fluorescent labels is CellMask orange.

2.4 Discussion and Conclusions

In this chapter, we discussed the principle of structured illumination microscopy and demonstrated the experimental realization of a DMD-based SIM system with super-resolution imaging capability. This DMD-SIM setup serves as the foundation of the whole dissertation. Despite the impressive performance of this standard DMD-SIM on 2D imaging, when it comes to high-dimensional data (xyz;c;t) acquisition, the imaging speed is significantly decreased due to the need to sequentially image through each dimension. As will be shown in the upcoming chapters, multiple innovations are going to be introduced that will ultimately allow this performing high-dimensional imaging in a "snapshot."

Appendix 2.A DMD control software

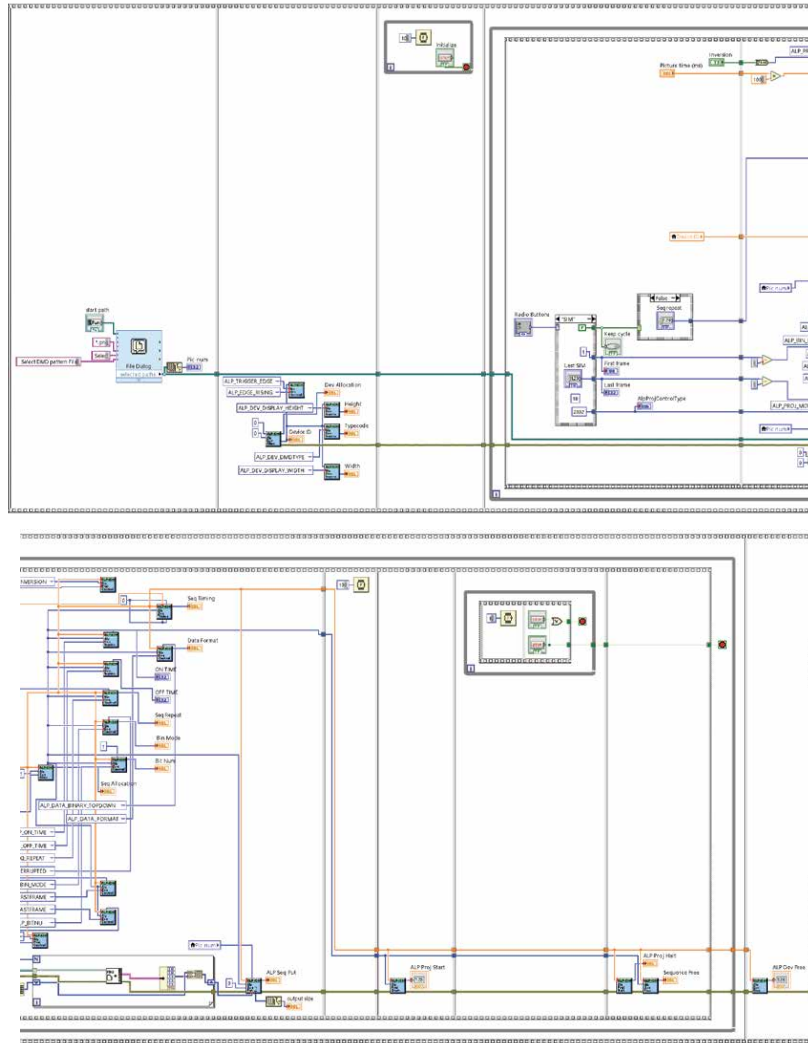


Figure 2.14: **The block diagram panel of DMD control LabView program.** The DMD is first connected and initialized, then the pattern sequence is uploaded and the parameters, including repeat number, picture time, etc, and the DMD can display the pattern sequence following the settings. After finishing displaying the sequence, the program can either release the DMD or restart the sequence again.

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CHAPTER 3

TACO: TANDEM ABERRATION CORRECTION OPTICS FOR SIM

3.1 Introduction

Advances in optical microscopy have played a key role in biological science research. More biological features and phenomena have been elucidated with better spatial resolution and techniques (Bond et al. (2022)). However, the quality of microscopy imaging is often hampered by optical aberration. The associated phase variations in the wavefront of the illumination and emission light from its ideal form arise from multiple factors such as index of refraction variations, sample scattering associated with the intrinsic inhomogeneity of complex biological samples, and misalignment and non-ideality of optics (Hampson et al. (2021)). These aberrations lead to decreased resolution, fidelity, and degraded signal-to-noise ratio (SNR) in microscopy images.

In recent years, adaptive optics (AO) has emerged as a promising approach to mitigate optical aberrations in microscopy (Booth (2014)). The idea is to add phase modulation opposite to the aberration using wavefront correctors such as deformable mirrors (DM) or spatial light modulators (SLM) (Ji (2017)).

Aberration correction with adaptive optics has been primarily applied to point scanning microscopy, e.g., confocal microscopy (Booth et al. (2002); Tao et al. (2011)) or multiphoton microscopy Ji et al. (2010); Wang et al. (2014), where a phase modulator is generally placed at the pupil plane, termed pupil AO, to correct aberrations that blur the illumination point and point spread function (PSF)(Mertz et al. (2015)). By contrast, aberration correction for wide-field illumination has been largely overlooked and considered to be of negligible importance because of the simplicity of the illumination form at the pupil plane. While this might be a reasonable assumption for uniform wide-field illumination (Booth (2014); Ji

(2017)), it does not hold true for structured illumination microscopy (SIM), which requires careful consideration of aberration correction beyond just the pupil plane.

SIM is a wide-field super-resolution imaging technique that utilizes patterned illumination (typically sinusoidal patterns) to down-modulate high spatial frequency information of a sample into low-frequency beat frequencies, also known as the Moiré effect (Gustafsson (2000)). High-resolution images can then be reconstructed by extracting high-frequency information from these beat patterns (Gustafsson (2000); Gustafsson et al. (2008)). However, the reconstruction process is very sensitive to errors in the patterns (Mudry et al. (2012)). Achieving robust reconstruction with minimal artifacts requires that a high-quality pattern be projected onto the sample.

Despite the aforementioned strict requirement for high-fidelity illumination patterns, SIM has long been considered immune to aberrations (Booth et al. (2015); Turcotte et al. (2019)). However, this assumption is only true if spatially-invariant aberrations are considered. Spherical aberration, astigmatism comma, and others have a global effect on the whole field of view (FOV) and can be represented by simple (e.g. parabolic) phase variation functions at the pupil plane of the optical system (Booth et al. (2015)). To illustrate, consider a sinusoidal illumination pattern that is formed by the interference of 2 or 3 coherent beams. The optical transfer function (OTF) of the pattern only consists of 2 or 3 wavevectors (points) in the pupil plane. For this reason, spatially-invariant aberrations will have a very limited effect on the illumination light, and only cause phase or orientation shifts to the illumination pattern. Consequently, almost all adaptive optics applications in SIM have focused on using pupil AO only effective in the detection path. Although an AO corrector could be placed at a shared pupil plane of both the illumination and detection path, it essentially has no effect on the illumination pattern and only corrects the detection path (Arigovindan et al. (2012); Débarre et al. (2008); Thomas et al. (2015); Turcotte et al. (2019); Li et al. (2020); Lin et al. (2021)).

However, in biological imaging, the aberrations introduced by the intrinsic heterogeneity of samples can cause severe distortion to a structured illumination pattern. Unlike spatially-invariant aberration, these sample-induced aberrations are spatially varying across the image plane (i.e. sample), and pupil AO can only correct a very limited field of view (FOV) (Mertz et al. (2015); Katz et al. (2012)). Despite its significance, this spatially-varying source of aberrations has been widely neglected. Clearly, a method to address spatially-varying aberrations in structured illumination is urgently needed.

One approach to address spatially-varying aberration is through conjugate AO (Mertz et al. (2015)), which refers to placing an AO corrector at a plane conjugate to the main source of aberration. Doing so directly maps the plane of phase modulation to the aberration plane and allows correcting the entire FOV with a single (but potentially complicated) correction function. The advantage of conjugate AO has already been extensively demonstrated (Mertz et al. (2015); Li et al. (2015); Park et al. (2015); Wu and Cui (2015); Amitonova (2018)). However, in contrast to pupil AO, conjugate AO cannot compensate for spatially-invariant aberrations such as spherical aberration, which turn out to be the most notorious aberration in the detection path of SIM (Arigovindan et al. (2012)). For this reason, achieving a fully aberration-corrected SIM system would be best achieved with a *tandem* aberration correction that combines the advantages of both pupil AO and conjugate AO.

In this chapter, we present a SIM microscope integrated with a novel Tandem Aberration Correction Optics (TACO) system that uses conjugate AO and pupil AO in the illumination path and detection path, respectively. A DM is used for conventional pupil AO in the detection path to correct for global aberrations, while an SLM is used to implement conjugate AO to compensate for local (spatially-varying) aberration in the illumination beam at the image plane. We demonstrate the effectiveness of our system on uniform fluorescent samples, fluorescent bead monolayers, and cell samples. Our results show that even in the presence of strong aberrations, our TACO system can robustly correct aberrations and greatly enhance

the quality of super-resolution reconstruction.

3.2 Adaptive optics for structured illumination

Consider the structured illumination formed by 3-beam interference, which is a configuration commonly used for super-resolution SIM imaging with optical sectioning (Gustafsson et al. (2008)). At the pupil plane, the illumination will form 3 diffraction-limited spots located at the center (0 order, $\rho = 0$) and periphery (± 1 orders, $\rho = \rho_0, \theta = \theta_0, \theta_0 + \pi$) as shown in Figure 3.1. Then after the objective, the three beams merge, interfere at the image i.e. focal plane, and form the periodic illumination pattern. In this process, the beams can be severely affected if traveling through a highly aberrating medium before reaching the image plane (Fig 3.1A).

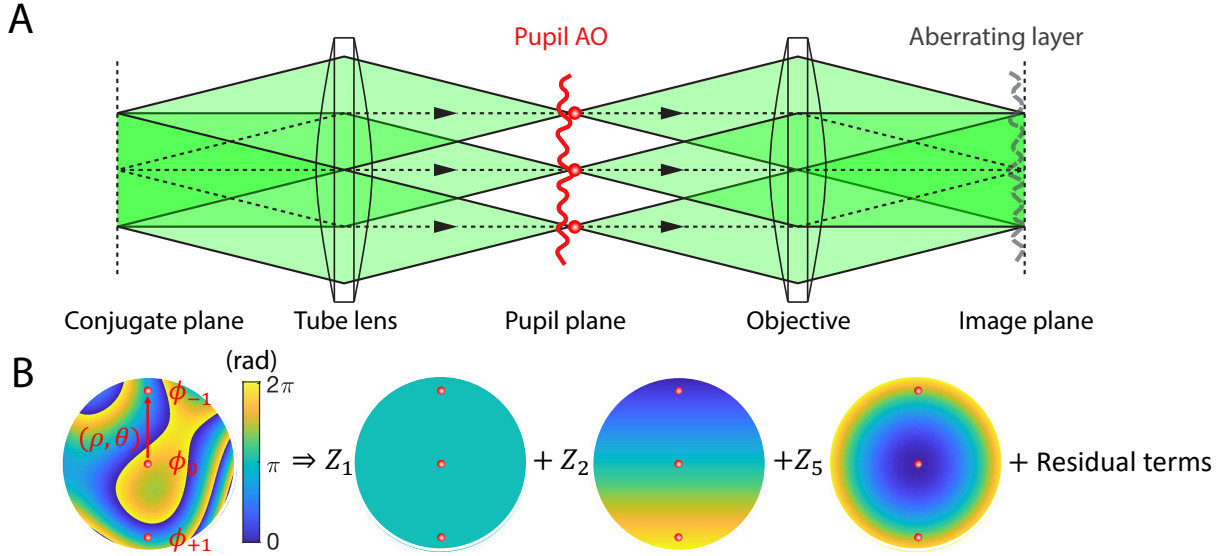


Figure 3.1: **Pupil AO aberration correction for structured illumination.** (A) Schematic of the working principle of pupil AO. The wavy red line represents the wave-front modulation at the pupil plane. (B) For 3-beam-interference structured illumination, any phase modulation at the pupil plane can be decomposed into a combination of piston, tilt, and defocus. The residual term has no effect on the illumination pattern. The three red points represent the 3 diffraction-limited spots of the illumination beam(s) at the pupil plane. The phase modulation exerted to the 3 points are denoted by $\phi_{0,\pm 1}$. The polar coordinate system of the pupil plane is denoted by (ρ, θ) .

If pupil AO was adopted, in which case a wavefront corrector (e.g., an SLM or a DM) is introduced for phase modulation at the pupil plane, then only those SLM pixels or DM segments that co-locate with the diffraction spots affect the phase modulation and the illumination pattern. However, the finite size of the SLM pixel or small local deformation of a DM results in essentially a constant phase modulation being applied to each diffraction order ($\phi_{0,\pm 1}$). Therefore, any phase modulation $\phi_p(\rho, \theta)$ applied to the pupil can be decomposed into the following form:

$$\phi_p = \frac{\phi_{-1} + 2\phi_0 + \phi_{+1}}{4} + \frac{\phi_{-1} - \phi_{+1}}{2} \frac{\rho}{\rho_0} \cos(\theta - \theta_0) + \frac{\phi_{-1} - 2\phi_0 + \phi_{+1}}{4} (2(\frac{\rho}{\rho_0})^2 - 1) + \text{res} \quad (3.1)$$

where the first three terms are known as the piston, tilt, and defocus Zernike modes Noll (1976). The combination of these three modes has the same effect on the illumination pattern as the original phase modulation $\phi_p(\rho, \theta)$ to the 3 diffraction-limited spots, and the residual terms can be neglected (Fig 3.1B). However, the effect of these three modes on the illumination pattern is trivial: they simply lead to a phase shift or orientation shift of the interference pattern (Booth et al. (2015); Liu et al. (2020)). Consequently, pupil AO can exert almost no effective pre-compensation to the illumination pattern to counteract subsequent distortion.

On the other hand, conjugate AO can address this problem rather straightforwardly. Consider a single aberrating layer with phase modulation $\phi_{ab}(\rho, \theta)$: conjugate AO employs a wavefront corrector placed in a plane conjugate with the aberrating layer to which one applies an opposite phase modulation of $\phi_c(\rho, \theta) = -\phi_{ab}$ and hence pre-compensate the illumination beam. In an ideal situation, the phase disruption from each small subregion of the aberration layer will be canceled out by the exact opposite phase modulation from the corresponding area of the wavefront corrector as shown in Fig. 3.2A&B. However, in practice, there are factors that limit the efficacy of conjugate AO. Firstly, the aberration may not arise from a single well-defined layer, which creates ambiguity regarding the optimal location of

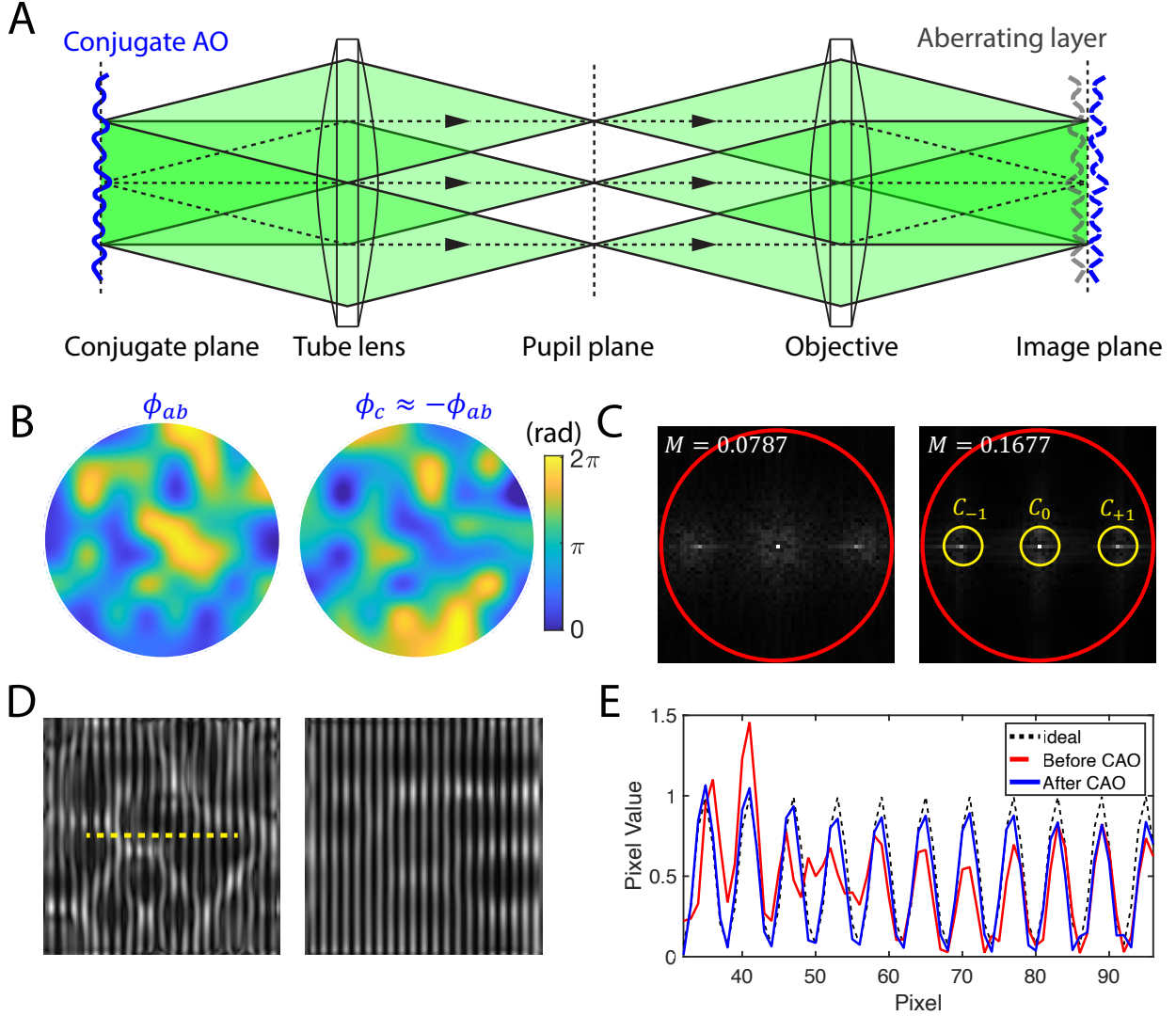


Figure 3.2: **Conjugate AO aberration correction for structured illumination.** (A) Schematic of the working principles of conjugate AO. The wavy blue line represents the wavefront modulation at the conjugate plane. (B) The simulated aberration phase mask $\phi_{ab}(\rho, \theta)$ (left) and the phase correction $\phi_c(\rho, \theta)$ (right). (C) The simulation results of the FT of the sinusoidal pattern before correction (left) and after correction (right), The yellow circles indicate the sub-images used for calculating the image quality metric M . The red circle indicates the pupil plane. (D) The simulation results of the sinusoidal pattern (intensity) before correction (left) and after correction (right). (E) The line-cut profile of the cross-section (dashed line) of the pattern in (D) before (red) and after (blue) conjugate AO correction, compared with the profile of an ideal pattern (dashed) without aberration.

the wavefront corrector. In the case of a thick aberration sample, multiple conjugate AOs (Bedggood et al. (2006); Simmonds and Booth (2013); Wu and Cui (2015)), or conjugate

AO with a refocusing system (Tao et al. (2017)), might need to be adopted. Secondly, the aberration ϕ_{ab} is usually unknown, and one needs to either directly measure it with a wavefront sensor (Li et al. (2015)), or optimize the AO phase mask in an iterative manner using an image quality metric (e.g., image contrast) that can be maximized or minimized (Mertz et al. (2015)). In this paper, we confine our discussion to the case of a single aberration layer from a scattering slide, but the TACO concept can be expanded to the case of multiple or thick aberration layers. We chose an image-based iteration method to avoid adding extra complexity to the optical apparatus. The image quality metric is defined as:

$$M = \frac{\sqrt{C_{-1}(w) \cdot C_{+1}(w)}}{C_0(w)} \quad (3.2)$$

where C is the contrast (here defined as the standard deviation) of the sub-images around diffraction spots of the FT of a raw image and w is a user-defined window to adjust the size of the sub-images (Fig. 3.2C). The contrast of the ± 1 pattern modulation spatial frequency wavevectors, $C_{\pm 1}$, serve as a criterion to allow quantifying the fidelity of the sinusoidal pattern. The 0-order spatial frequency feature, C_0 , in the denominator serves as a normalization to exclude the erroneous solution of increasing the overall brightness of the whole image.

To test the validity of the image quality metric M , we simulated conjugate AO correction for SIM illumination. A random phase mask was introduced near the image plane of a 4-f imaging system (Mertz (2019)) to cause aberration, and the phase modulation was iteratively updated at the conjugate plane to counteract the aberration by maximizing the value of the metric as shown in equation 3.2. The iteration method is based on a stochastic parallel gradient descent (SPGD) algorithm (Vorontsov and Sivokon (1998); Bing Dong and Ji Yu (2015)). During each iteration, small random perturbations are applied to the phase mask that causes a change of the metric value of δM . The phase mask is subsequently updated using δM as an estimation for the average gradient of M versus the phase mask values. The

details of the algorithm are provided in the Supplementary Document. The value of the metric of the initial pattern is 0.0787. After 500 iterations, the metric value converged to a stable value of 0.1677 (Figure S1). The resulting phase modulation, ϕ_c , at the conjugate plane shows a complementary distribution compared to aberration ϕ_{ab} as shown in Fig. 3.2B, which indicates that most of the spatially-varying aberration has been canceled out by conjugate AO. After correction, the first-order spot of the Fourier Transform (FT) of the pattern shows a $\sim 1.5\times$ increase of intensity versus no correction, while the zero-order spot remains the same (Fig. 3.2C). Figures 3.2D & E show the intensity of the pattern before and after AO correction. Although the pattern has initially been severely distorted by phase aberration, the conjugate AO approach just declared has almost completely compensated the aberration such that the fidelity of the modulation is almost identical to the ideal sinusoidal unaberrated pattern.

3.3 Experimental method

3.3.1 Microscopy setup

We built a structured illumination microscope (SIM) integrated with a TACO system to illustrate the comprehensive aberration correction capability. An illustration of the TACO-SIM optical setup is shown in Figure 3.3A. A 3-dimensional rendering that closely resembles the actual apparatus is shown in Figure 3.3B.

A solid-state CW laser (Millennia Vs, Spectra Physics, 532 nm, 5-watt maximum power) was used as an illumination light source. The intensity and polarization of the laser beam are controlled by a pair of linear polarizers (P1, P2) and a half-wave plate (HWP1). The laser light passes through a $4\times$ beam expander formed by a pair of achromatic lenses (L1, L2). A diamond pinhole was placed at the focus to clean the beam profile. A spatial light modulator (Hamamatsu LCOS-SLM X10468) was used for wavefront correction in the illumination

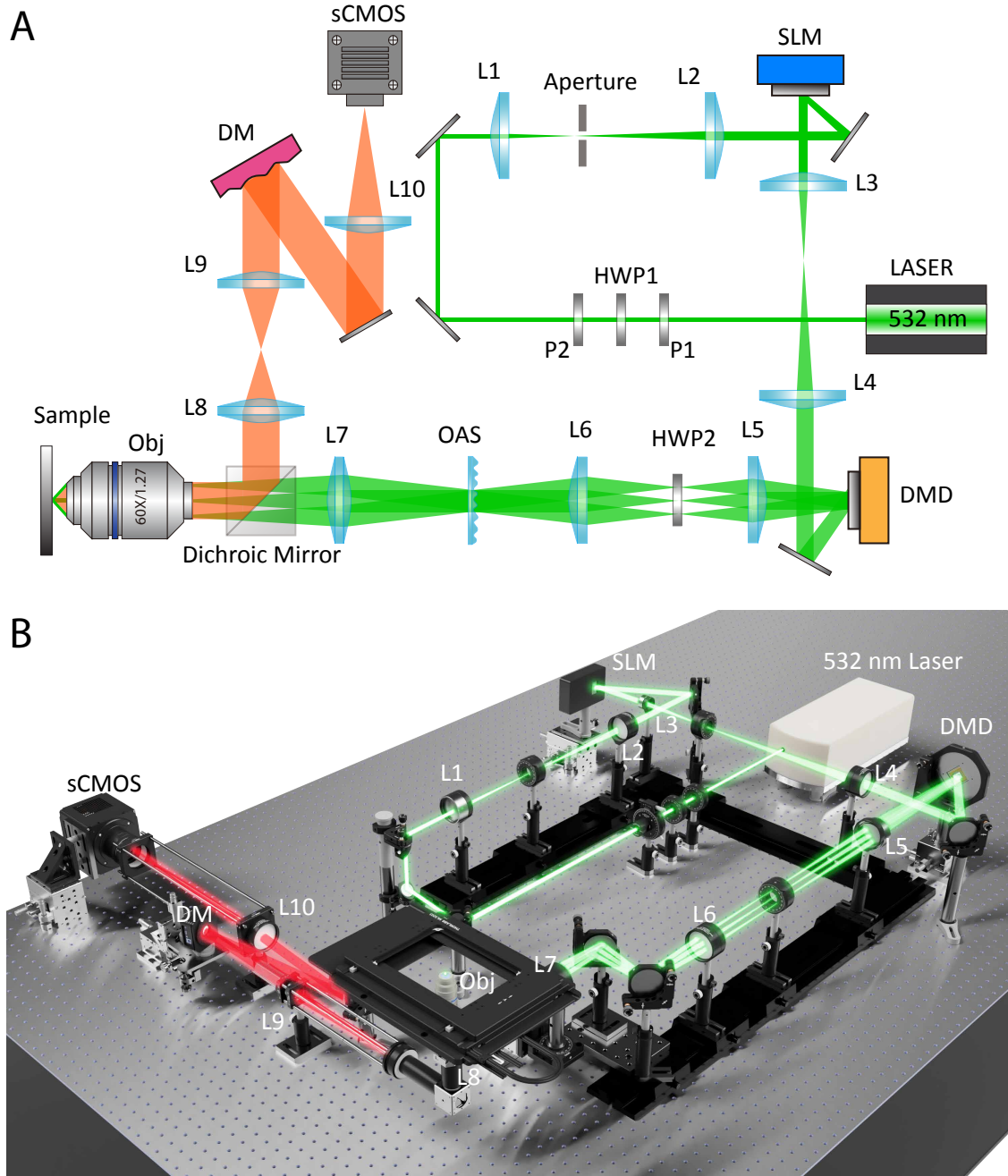


Figure 3.3: Optical (hardware) setup of the TACO-SIM system. (A) Schematic representation of the microscopy hardware setup. HWP1-HWP2, half-wave plate; DM, P1-P2, linear polarizers. OAS, optical aberration slide; Obj, objective lens; L1-L10, lenses. Lens focal lengths: $f_1 = 100$ mm, $f_2 = 400$ mm, $f_3 = 100$ mm, $f_4 = 400$ mm, $f_5 = 200$ mm, $f_6 = 250$ mm, $f_7 = 300$ mm, $f_8 = 200$ mm, $f_9 = 200$ mm and $f_{10} = 400$ mm. (B) 3D rendering of the optical setup. See text for details.

path. We used an SLM instead of a DM to allow very detailed phase information to be encoded in the 800×600 8 bits pixels of the SLM compared to a deformable mirror (<100 piezo actuators), which allows much more exquisite wavefront modulation to compensate for spatially-varying aberrations. The coherent beam reflected from the SLM is relayed to a digital micromirror device (Texas instrument, DLP9000X VIS WQXGA) by a pair of lenses with $4\times$ magnification (L3, L4) to cover the entire size of the DMD. The digital micromirror device (DMD) and its corresponding controller board (Digital Light Innovations DLP V4390) were used to generate the structured illumination patterns (see Figure S2). The DMD is rotated 45° in order to make the input and output beams lie in a common horizontal plane. A detailed description of the optical setup of the DMD is provided in the Supplementary Document. The 3 diffraction beams resulting from the DMD are imaged to the back focal plane of the tube lens (L7) by a pair of relay lenses with $5/4\times$ magnification (L5, L6). A half-wave plate (HWP2) mounted on a piezo rotation stage (Newport AG-PR100) is placed at the focal plane to adjust the polarization of the beams in order to maximize the interference contrast, (O'Holleran and Shaw (2012)). An optical aberration slide (OAS) is placed at the back focal plane of L7 to introduce spatially-varying aberrations in the illumination path. The OAS was made by manually spreading transparent nail polish (Electron Microscopy Science) on a pre-cleaned slide (Fig. 3.4), which is the same method reported previously (Sohmen et al. (2023)). After propagating through the OAS, the now aberrated 3 coherent diffraction beams are directed to the backport of a Nikon Ti Eclipse microscope body, focused by L7 to the pupil plane (back aperture) of the objective (SR Plan Apo, $60\times$, 1.27 WI, Nikon), and form an interference pattern at the sample plane. For simplicity of demonstration, the OAS only affects illumination light and the emission light is unaffected, so the SLM and conjugate AO only need to operate in the illumination path to compensate. However, this setup with the OAS already manifests significant disruption of the SIM pattern allowing us to demonstrate the capability of the TACO system. The

scheme can be extended to the case when the aberration medium affects both illumination and emission light by placing a wavefront corrector in (a common image plane shared by) both illumination and emission paths.

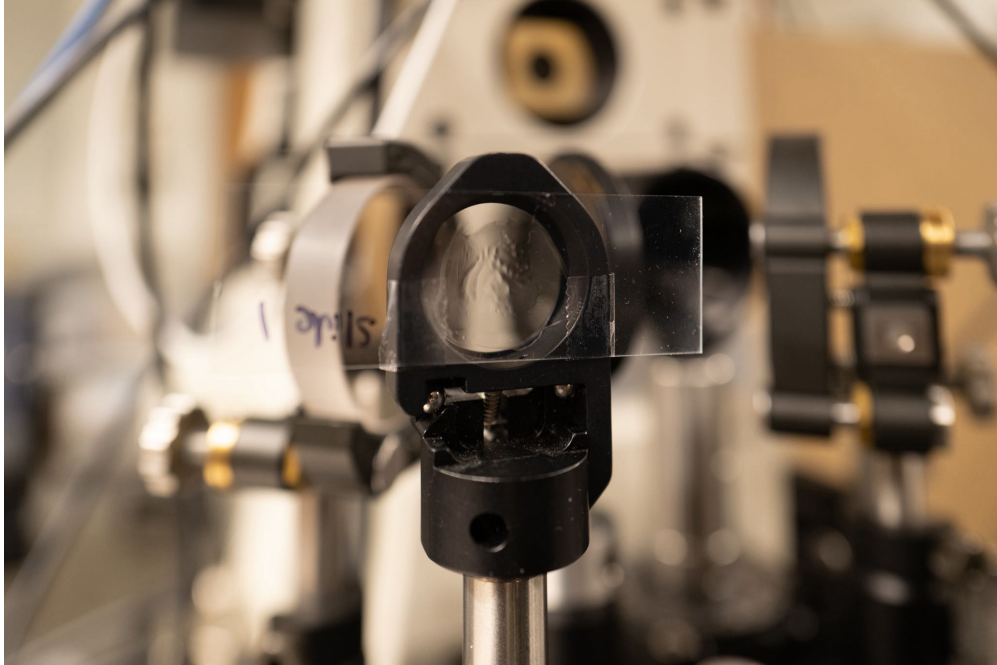


Figure 3.4: Photo of the optical aberration slide in the illumination beam path at the location shown in the experimental schematic in Fig. 3A. The slide is placed at the back focal plane of the tube lens L7, and imaged to the image (sample) plane by the tube lens and objective to introduce spatially-varying aberrations.

A high-speed sCMOS array detector (Photometrics Kinetix, $6.5 \mu\text{m}$ pixel size) is used to record images. A pair of lenses (L9, L10) with a magnification of $2\times$ relay the emission from the output port of the microscope to the detector, hence each pixel in the image corresponds to 54 nm at the sample plane. The reason to have the $2\times$ relay lens pair is to magnify the FOV of the 16 mm c-mount output port to fully cover the large 29.4 mm detector sensor. Although in the present work, we do not really need this large magnification and small pixel size, full usage of the large sensor size is necessary for our other research that uses the same microscope-detector setup. A deformable mirror (Thorlabs DMH40-P01) was placed at the pupil plane for aberration correction in the detection path. There are several reasons for

using a DM instead of an SLM in the detection path. Firstly, fluorescent light is unpolarized, while the SLM only works for light with a certain linear polarization. Secondly, the phase modulation of the SLM (on light) is wavelength-dependent, so a fluorescent sample, which has a broad emission spectrum, will result in chromatic aberration if using an SLM. Thirdly, although a DM has less detailed phase modulation capacity compared to an SLM, it is sufficient for global aberration correction at the pupil plane. Finally, the update rate of the DM (4 kHz) is much higher than any SLM (e.g., 60 Hz), allowing additional functions such as high-speed focal plane scanning along the z-axis by changing the curvature of the DM to defocus and refocus the emission light.

3.3.2 Tandem Aberration Correction Optics (TACO)

Implementation of the TACO method is conducted in 2 steps. The first is an image-based pupil AO iteration using the DM to correct global aberrations manifest in the detection path. During this process, the DMD is set to present a completely uniform image (i.e., uniform illumination) to enable wide-field imaging without any structured illumination. The deformation (shape) of the DM is defined by a linear combination of the first 15 Zernike polynomials Noll (1976). The iteration is based on the SPGD algorithm (as described later) and the metric is the contrast of the image. Here the contrast is defined as the standard deviation $\sqrt{\frac{1}{N} \sum_i^N (I_i - \bar{I})^2}$ (N : total number of pixels, I_i : value of the i_{th} pixel, $\bar{I}$: mean pixel value) of the image. In each iteration step, the Zernike coefficients will be updated to reshape the DM. Figure 3.5A shows a typical AO process of the DM on images of fluorescent beads. The aberration was introduced by the misalignment of optics. At the beginning of the iteration, the image was severely blurred, while an improvement in image quality can be observed (Fig. 3.5A, upper) as the DM's shape is adapted to counteract the optical aberration (Fig. 3.5A, lower left). It typically takes $50 \sim 100$ steps for the image contrast to converge to a plateau (Fig. 3.5A, lower right).

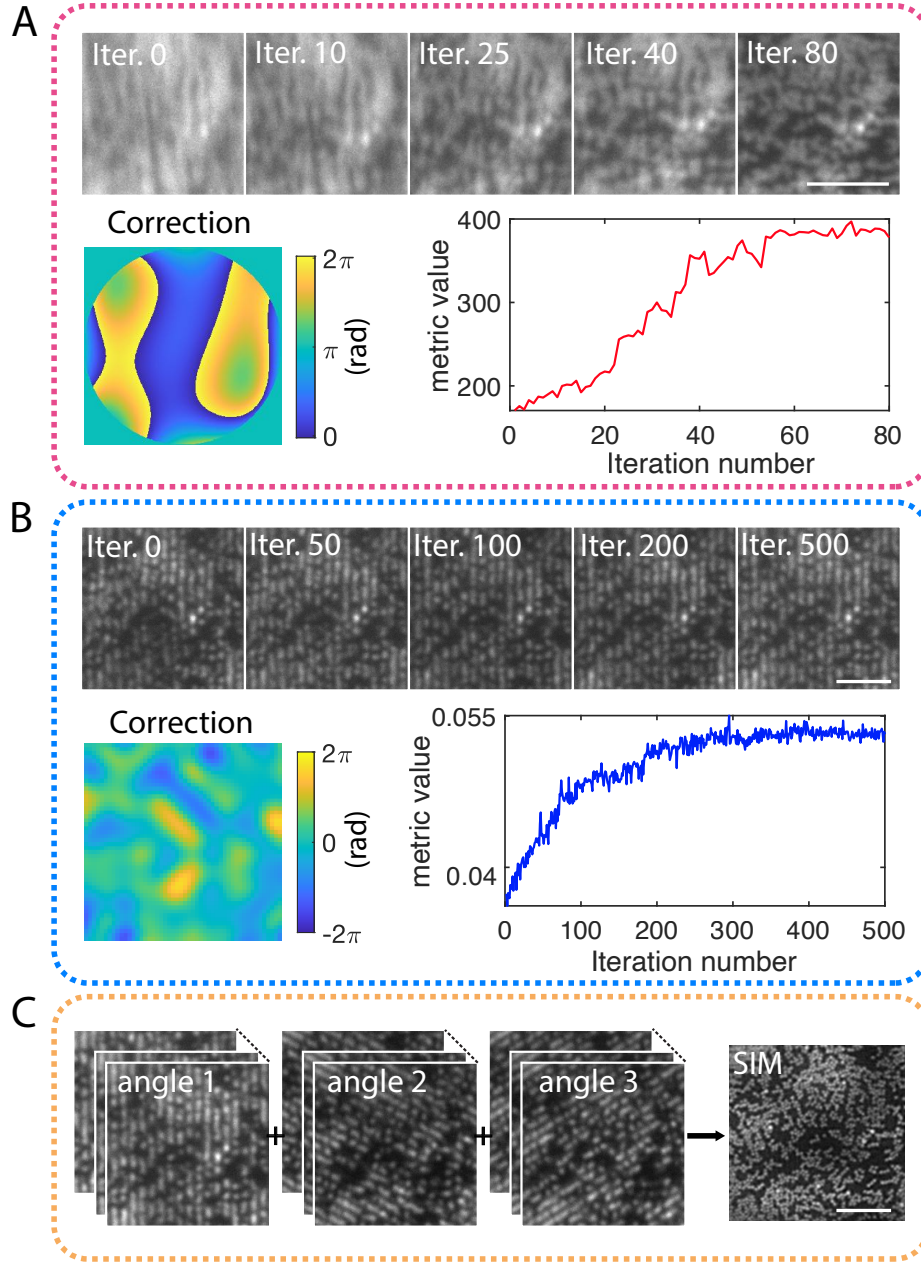


Figure 3.5: **Workflow of the TACO-SIM system.** (A) AO iteration process of DM. Upper: Images of fluorescent beads at different iteration steps; Lower left: final wavefront correction pattern of DM; Lower right: the metric value of images versus iteration number. (B) AO iteration process of SLM. Upper: images of fluorescent beads superimposed with a SIM pattern at iteration steps; Lower left: final wavefront correction pattern of SLM. The phase modulation has been unwrapped to better visualize the phase topography; Lower right: the value of the metric of the images versus iteration number. (C) SIM reconstruction from 3 sets of images with SIM pattern at different angles. Scale bars in A, B & C: $4\ \mu\text{m}$.

The second step is an image-based conjugate AO iteration using the SLM to correct spatially-varying local aberrations of the illumination path. In this step, the DMD is programmed with a binary periodic pattern for structured illumination. The wavefront modulation of the SLM is defined by values of a 600×600 -pixel array. The metric M is defined in Eq. 3.2 and the iteration procedure is based on the SPGD algorithm. Before each update, a Gaussian filter with an empirically determined standard deviation (usually ranging from 0.75 to 1.25) is applied to the SLM pixel array, in order to match the smoothly varying phase topography of the aberration slide. Theoretically, the phase correction can be achieved by optimizing pixel-by-pixel independently, but this requires many iterations. The use of an appropriate and modest Gaussian kernel substantially reduces the iterations required. For phase modulations out of the $0 - 2\pi$ range, the pixel values are wrapped at 2π (pixel value = 224 in our case) to fit in the modulation range of the SLM. The illumination beam propagates through the aberration slide and becomes strongly distorted. Figure 3.5B shows a typical AO iteration process of the SLM on the fluorescent beads images. It can be seen that although the AO correction by the DM enables functional wide-field imaging with uniform illumination, the structured illumination pattern shows severe distortion resulting from the aberration slide. This distortion was largely rectified (Fig. 3.5B, upper) as the SLM updated its pixel array values to compensate the aberration (Fig. 3.5B, lower left). Typically it takes $300 \sim 1000$ iterations for the metric to converge (Fig. 3.5B, lower right).

3.3.3 SIM imaging and reconstruction

We use a series of striped illumination patterns formed by the DMD (Fig. S2) to generate structured illumination. The three-beam interference configuration is employed for its optical sectioning capability (Gustafsson et al. (2008); Müller et al. (2016)) when imaging 3D samples like cells. The pattern period is 6 pixels and results in an (up to) 85% improvement in resolution with very good pattern contrast maintained. The $1.85\times$ resolution improvement

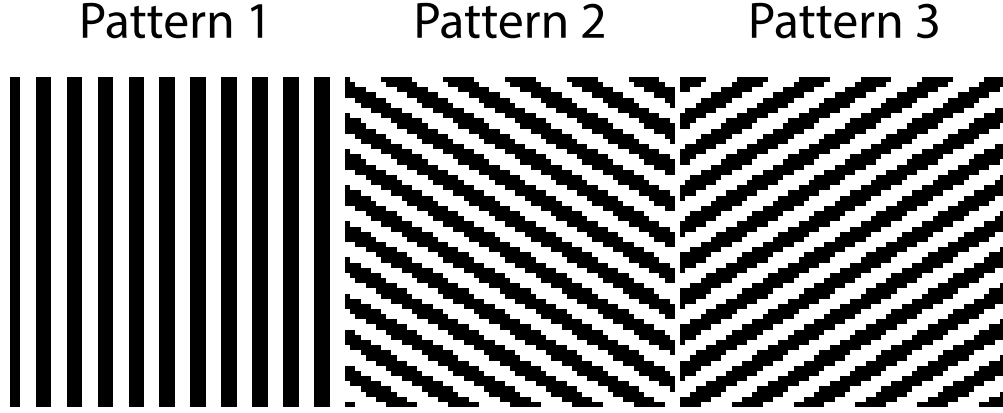


Figure 3.6: **Representative (portions of) patterns for the DMD to generate SIM illumination.** The patterns are binary stripes oriented at 0° , -60° , and 60° with a 6-pixel period (3 on, 3 off). During one SIM imaging process, the patterns are projected by the DMD (2560×1600 pixels) onto the sample plane in a time series. The second-order spatial frequency of the structured illumination patterns at the sample plane is $3.07\mu\text{m}^{-1}$ (i.e., 326 nm period), which is the key to down-modulate the high-frequency information and increasing imaging resolution. The calculation of the theoretical resolution improvement depends on the choice of the definition of the microscopy resolution. With the emission wavelength $\lambda_{em} = 580$ nm and a 1.27 NA objective lens being used, the spatial resolution of the wide-field imaging is 228 nm by the Abbe limit ($d = \lambda_{em}/2NA$), or 278 nm by the Rayleigh criterion ($d = 0.61\lambda_{em}/NA$). These two definitions of WF resolution are $0.7\times$ or $0.85\times$ of the SIM pattern period. The theoretical resolution improvement of the SIM is correspondingly $1.7\times$ or $1.85\times$, depending on which definition of WF resolution is compared to. The FairSIM reconstruction software adopts the Abbe limit. However, from the FRC analysis of the images of point-like-sources (20 nm fluorescent beads), the resolution improvement obtained is closer to 1.85 (Fig. 3.7)

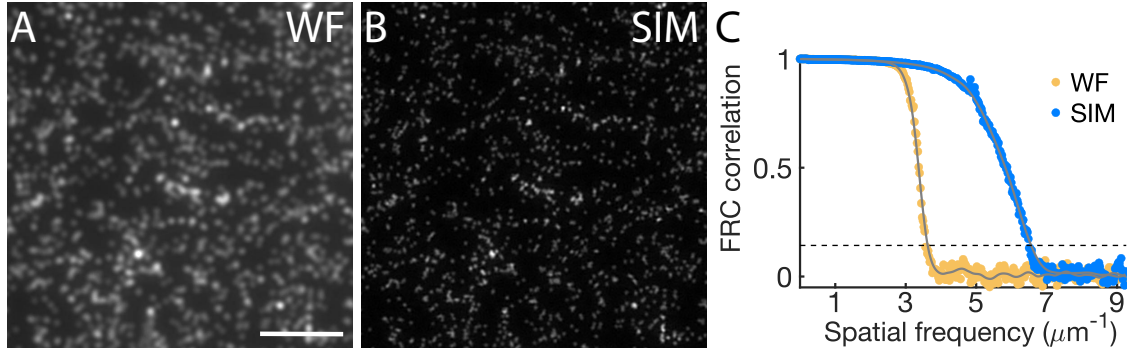


Figure 3.7: **Fourier Ring Correlation (FRC) measurement of 20 nm diameter fluorescent beads sample.** (A) Wide field (WF) image of the 20 nm beads sample. (B) SIM reconstruction image of the 20 nm beads sample. (C) FRC curves of the WF image and the SIM image. The FRC analysis is conducted based on the method described in Nieuwenhuizen et al. (2013). 10 different fields of view (FOVs) were analyzed. For each FOV, two sets of images with the same parameter settings were acquired independently for FRC curve calculation. The image resolutions were determined when the FRC curves dropped below a fixed threshold of $1/7$. This leads to a wide-field resolution of 282 ± 4 nm, and a SIM resolution of 153 ± 2 nm, corresponding to a resolution improvement of 1.84 ± 0.03 .

is determined by both theoretical calculation (Fig. 3.6) and Fourier ring correlation (FRC) analysis on the experimental images of 20 nm fluorescent bead samples (Fig. 3.7). Figure 3.5C shows a set of typical SIM raw images and the associated reconstruction result. 18 patterns with $3 \text{ angles} \times 6 \text{ phase shifts}$ are used to create the complete image set required to reconstruct a SIM image. The reason for using 6 phases instead of the conventional 5 phases is to achieve an equidistant phase for the 6-pixel period of the phase patterns. FairSIM, an ImageJ-based open-source SIM reconstruction software (Schneider et al. (2012); Müller et al. (2016)), is used for the reconstruction. Figure 3.5C shows an example of 18 raw images of 150×150 pixels reconstructed into super-resolution SIM of 300×300 pixels, with each pixel corresponding to 27 nm effective pixel size in the image. Here the dimension of 150×150 pixels is purely for display purposes. The other SIM reconstruction images presented in this paper are larger in size. Note that FairSIM uses the OTF attenuation method to retain the optical sectioning ability, which is not a full 3D reconstruction method. More details of the SIM reconstruction can be seen in the Supplementary Document.

3.4 Experimental results

3.4.1 TACO correction for SIM patterns

The TACO system was first tested on a nearly uniform thin sample layer of carboxylate-modified polystyrene fluorescent beads (Fig. 3.8). As shown in Fig. 3.9A and 3.9B, a SIM pattern without any aberration was imaged, then aberrations were introduced from the aberration slide and also from misalignment of optics. Comparison of Figs. 3.9 A & B shows that the sinusoidal pattern was severely distorted by the aberration of the optical beam (Fig. 3.9B1). This is also manifest in the Fourier spectrum since the ± 1 diffraction orders are barely discernible (Fig. 3.9B2).

Figure 3.9 C shows the result of only using the DM and pupil AO correction in the detection path. This DM correction resulted in better overall sharpness and contrast in the sinusoidal modulated pattern and its FT (Fig. 3.9F). However, the spatially varying distortion of the pattern still persists. (Note that a spotted grid-like pattern is evident. This results from the SLM pixelation, and also causes the minor diffraction orders in the FTs of the patterns. These grid-like patterns are more obvious when there are spatially varying aberrations present.) To address the spatially-varying aberration, a tandem stage of conjugate AO correction was conducted using the SLM. After 500 iterations, the pattern was restored nearly to the ideal unaberrated quality (Fig. 3.9D). After SLM correction, the profile of the pattern has a shape and contrast close to the ideal undistorted case, which is a significant improvement in comparison with the case of DM-only correction (Fig. 3.9E). The corresponding FTs also show that the diffraction orders following the implementation of TACO become much sharper compared with DM-only correction.

We also tested the same SLM correction on patterns with different orientations as required for SIM reconstruction and found that the quality of all the sinusoidal patterns (i.e., all 3 orientations) were significantly improved (Fig. 3.10). This observation indicates that the

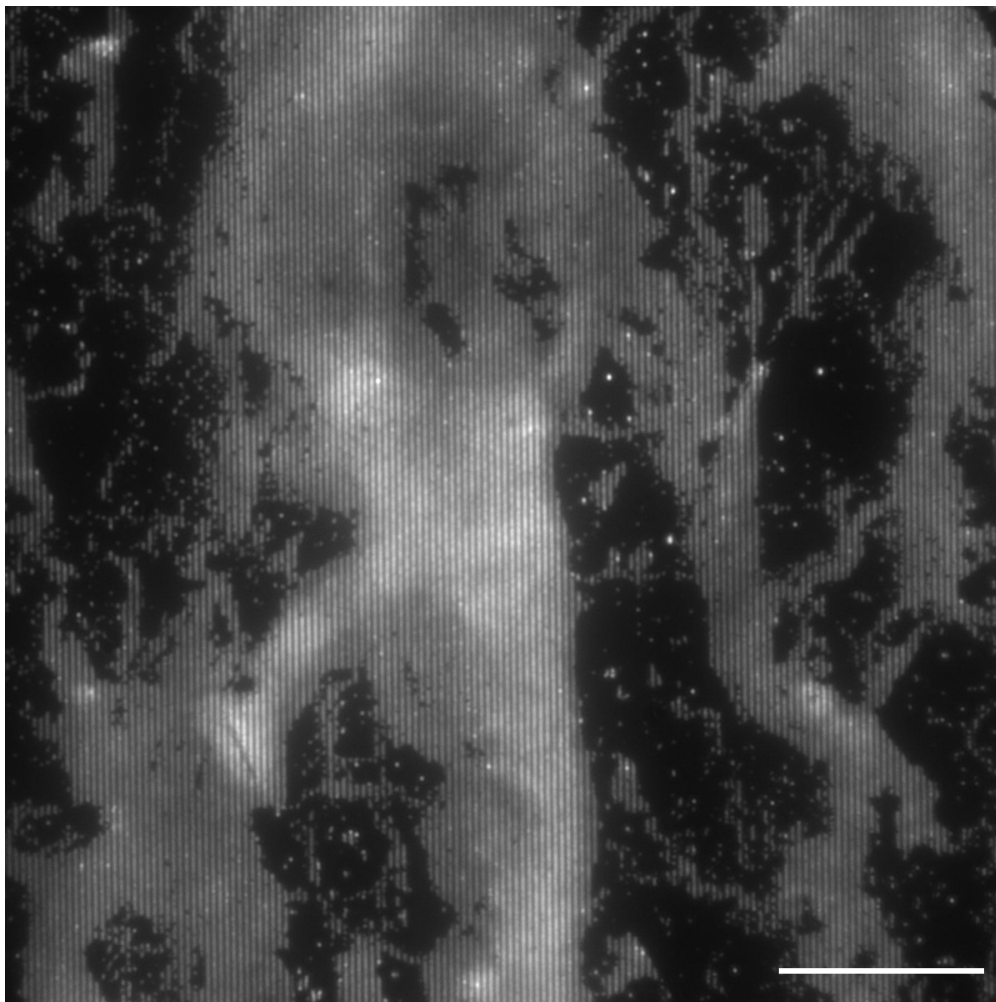


Figure 3.8: A larger field of view (1800×1800 pixels) of the 200 nm dia. bead layer sample shown in Figure 5 with structured illumination from the DMD. Scale bar: $20 \mu\text{m}$.

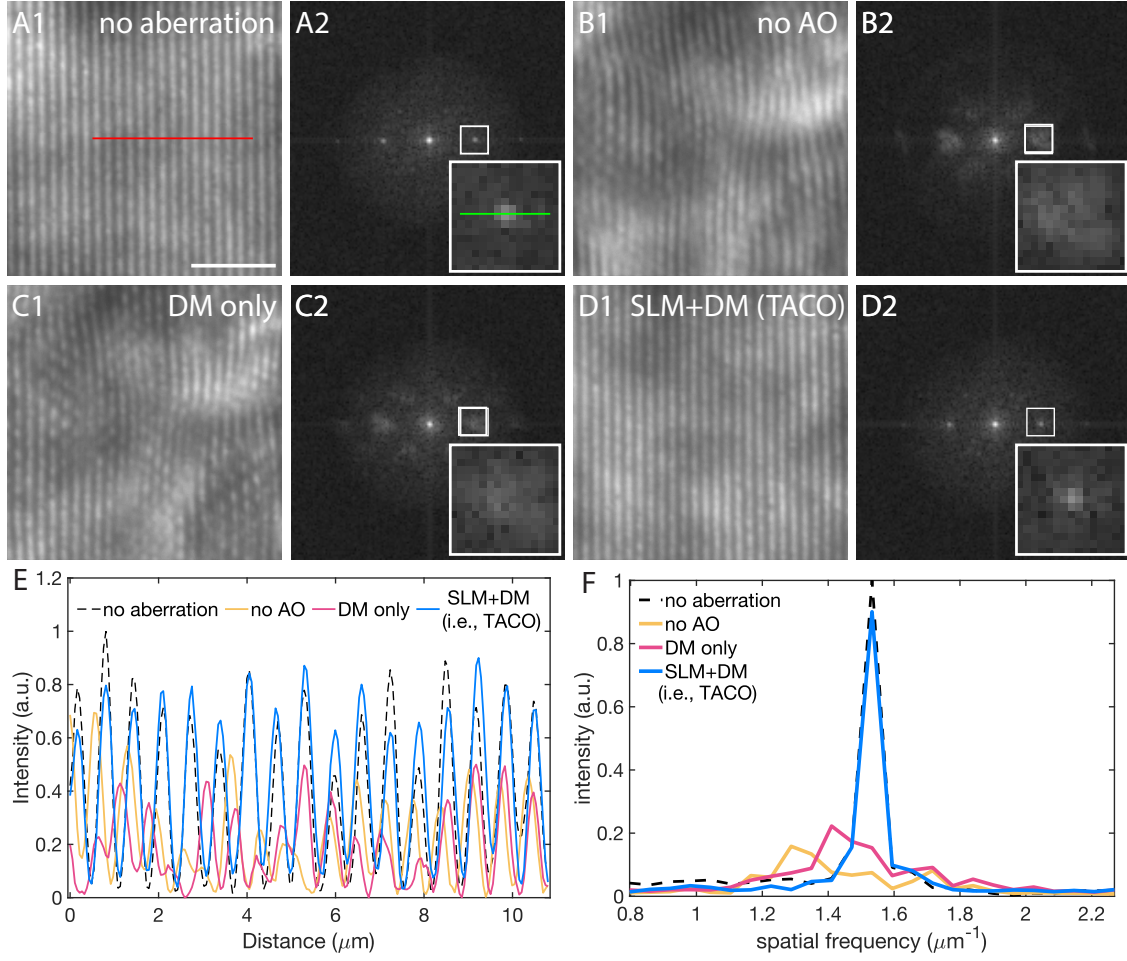


Figure 3.9: Experimental structured light patterned samples and their Fourier Transforms. (A-D) SIM patterns and associated FT for different conditions. (A1 & A2) no aberration was introduced. (B1 & B2) aberration was introduced both globally and locally. (C1 & C2) Only pupil AO correction by the DM was performed. (D1 & D2) Both conjugate AO by the SLM and pupil AO by the DM were performed, which is the full implementation of TACO. (E) Intensity profile of a cross-section (red line in A1) of the illumination pattern for different conditions. Each curve is smoothed by a moving average with a 3-pixel window and subtracted by the image background to reach the same baseline. (F) Intensity profile of a cross-section (green line in A2) around the first-order spot of FTs for different conditions. To mitigate the edge artifacts, a Gaussian window with a width factor of 0.6% of the image size was applied to the images before doing the Fourier transform. All the FTs have been scaled by a factor of 2, and Gamma corrections are applied with $\gamma = 0.2$. Scale bar in A: 5 μm . Effective pixel size: 54nm.

correction is not just limited to a specific pattern orientation. Rather, the wavefront is restored to a plane wave, so optimization of a single SIM pattern gives an overall correction

to all the sinusoidal patterns. Doing the SLM correction process based on the image quality of multiple angled patterns can also lead to even more thorough improvement, but comes at the cost of 50-100 additional iterations (Fig. 3.11).

3.4.2 *TACO-SIM imaging of fluorescent beads*

We tested the quality of SIM reconstruction using images of a sparsely distributed 2D 200 nm dia. fluorescent bead sample. Fig. 3.12A shows a wide-field image of a slide of red fluorescent beads. The 200 nm diameter of the beads is smaller than the resolution of our wide-field microscopy system. The bead sample was illuminated with SIM patterns with different amounts of aberration and different types AO corrections, and reconstructed into the corresponding super-resolved SIM images (Fig. 3.12B-F). In Figure 3.12B, the SIM pattern was corrupted by both spatially-varying aberration and global aberration, while no AO correction was performed. The resulting image reconstruction is poor, showing very pronounced artifacts. In Fig. 3.12C, only SLM aberration correction for the excitation path was performed, showing some improvement. However, due to the existence of global aberration, the profile of individual beads is still distorted. In Fig. 3.12D, only detection path aberration correction was performed using the DM. The quality of reconstruction is spatially-dependent: in some areas, the reconstructed image is free of artifacts and shows good quality (e.g, at the center of the image), while in other areas (e.g., the upper left and lower right parts), the image quality is still severely deteriorated. This result shows how spatially-dependent aberration corrupts image reconstruction in a different way than global aberrations. Finally, Fig. 3.12E shows a substantial improvement with full aberration correction, i.e. implementation of TACO. In Fig. 3.12F, no aberration was introduced, and the image was the most ideal. We can see that the image quality after full TACO correction is very close to the ideal unaberrated condition of Fig. 3.12F

Fig. 3.12G shows the intensity profile of bead 2 as shown in the inset. The profile

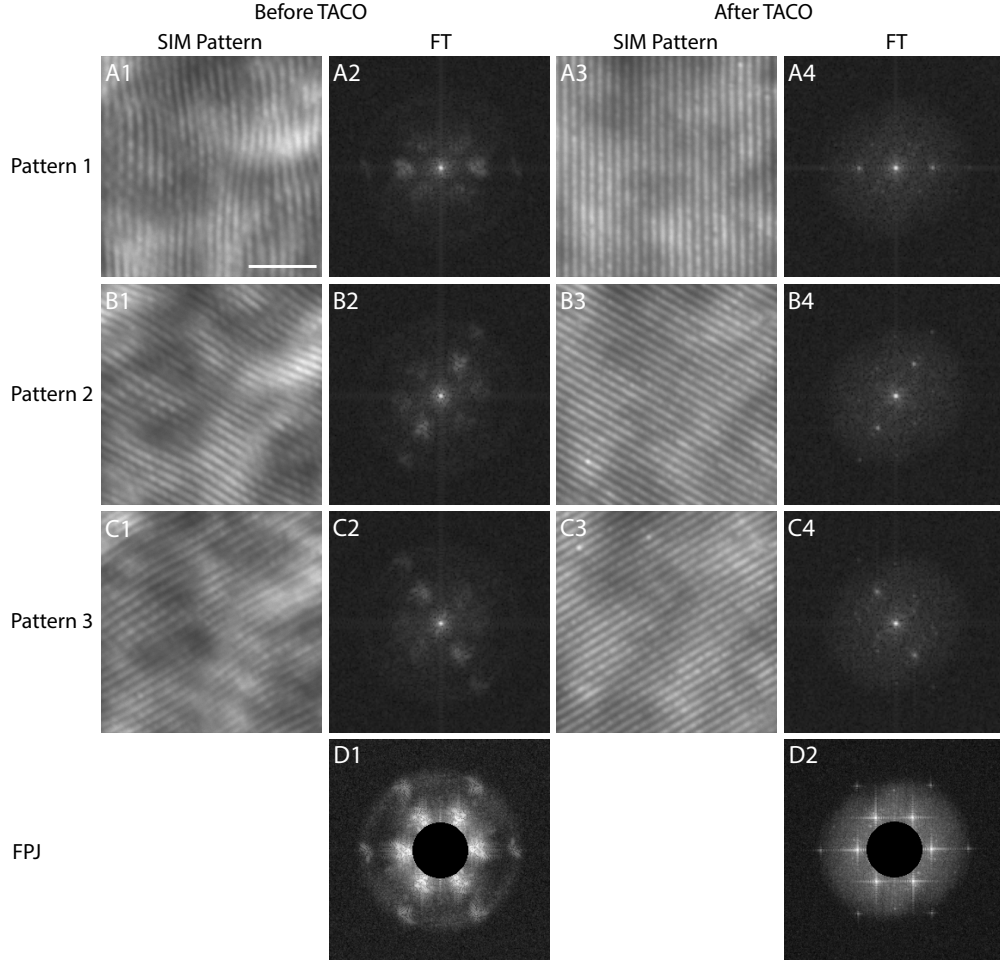


Figure 3.10: **One SLM correction iteration series can enhance all angled patterns.** An iterative SLM-based aberration correction process was conducted based on pattern 1. The resulting SLM phase mask counteracted the aberration introduced from the optical aberration slide and led to a significant improvement in pattern quality at the image plane as well as the sharpness of the diffraction orders in the FT spatial frequency images (A1-A4). The same SLM phase mask also substantially recovered the quality of pattern 2 (B1-B4) and pattern 3 (C1-C4). (D1-D2) Raw Fourier Projection (FPJ) of the FTs of the entire 3×6 image set before and after TACO correction. The generation of FPJs is based on (Ball et al. (2015)). With the low-frequency parts being masked, FPJs provide better visualization of the higher diffraction orders. In the FPJ after TACO correction, both first-order and second-order spots show greatly narrowed distributions. This result illustrates that the aberration correction from the SLM is not biased to any single structured illumination pattern. Instead, it corrects the whole wavefront towards an ideal plane wave. Scale bar in A1: $5 \mu\text{m}$.

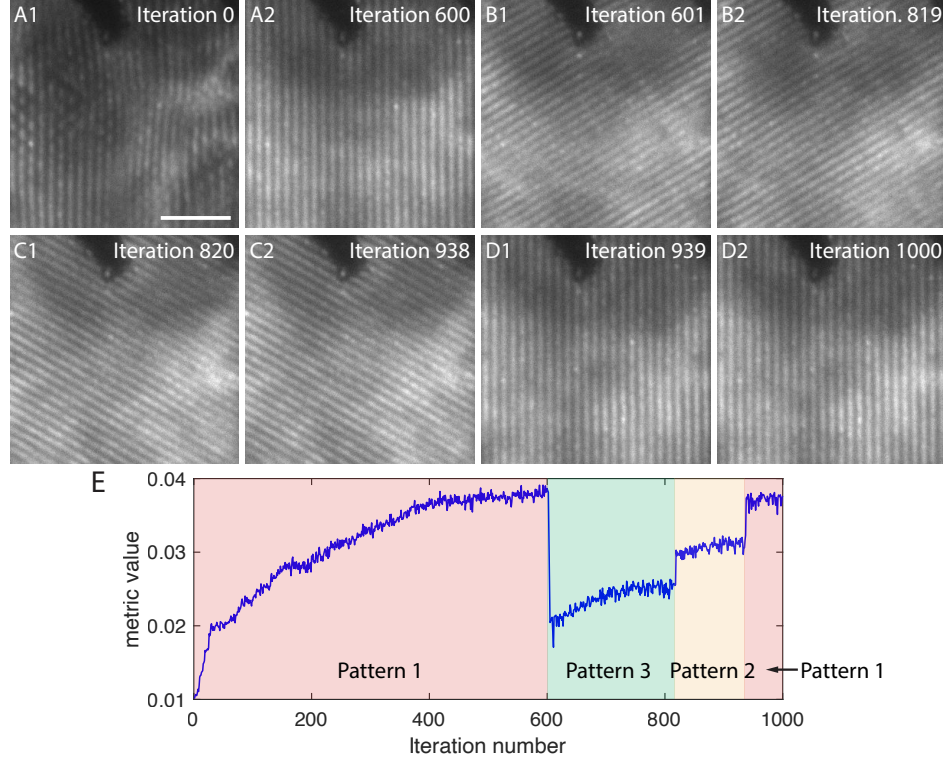


Figure 3.11: **Illustration of an iterative SLM-based aberration correction process using multiple structured illumination patterns.** The iteration process is divided into 4 stages. Stage 1 is based on pattern 1 (A1, A2). During this stage, the image quality metric defined in equation 2 plateaus to a (relative) maximal value by 600 iterations (E). Stage 2 is based on pattern 3 (B1, B2). Stage 3 is based on pattern 2 (C1, C2). In stage 4, the pattern is switched back to pattern 1 and the iteration continues around a hundred rounds to determine if the associated metric value is also converged (D1, D2). Despite stage 1 resulting in an overall correction to all three patterns, stages 2 & 3 do complement the optimization in stage 1 (pattern 1) and slightly further increase the image metric values (E). The image metric values shown in (E) manifest jumps and offsets when the pattern is switched because of the difference in intensity distribution and contrast between different patterns. Scale bar in A1: 5 μm . The further improvement of the metric value upon changing the pattern reflects an inherently positive nature of our optimization methodology. When the pattern angle is altered, new information about the wavefront is introduced, enabling further correction of aberrations. The plateau observed in the pattern 1 phase signifies a substantial correction of aberrations based on the information available from that particular pattern. However, as each pattern provides unique insights into the wavefront, switching to patterns 2 and 3 introduces additional, complementary information that enhances the correction process. Ideally, additional iterations between these patterns would lead to an increasingly optimal wavefront correction. In practice, however, we are limited by factors such as photobleaching, so we limited the total iteration steps to a maximum of 1000. As shown in the figure, this approach still yields a high-quality correction for SIM patterns.

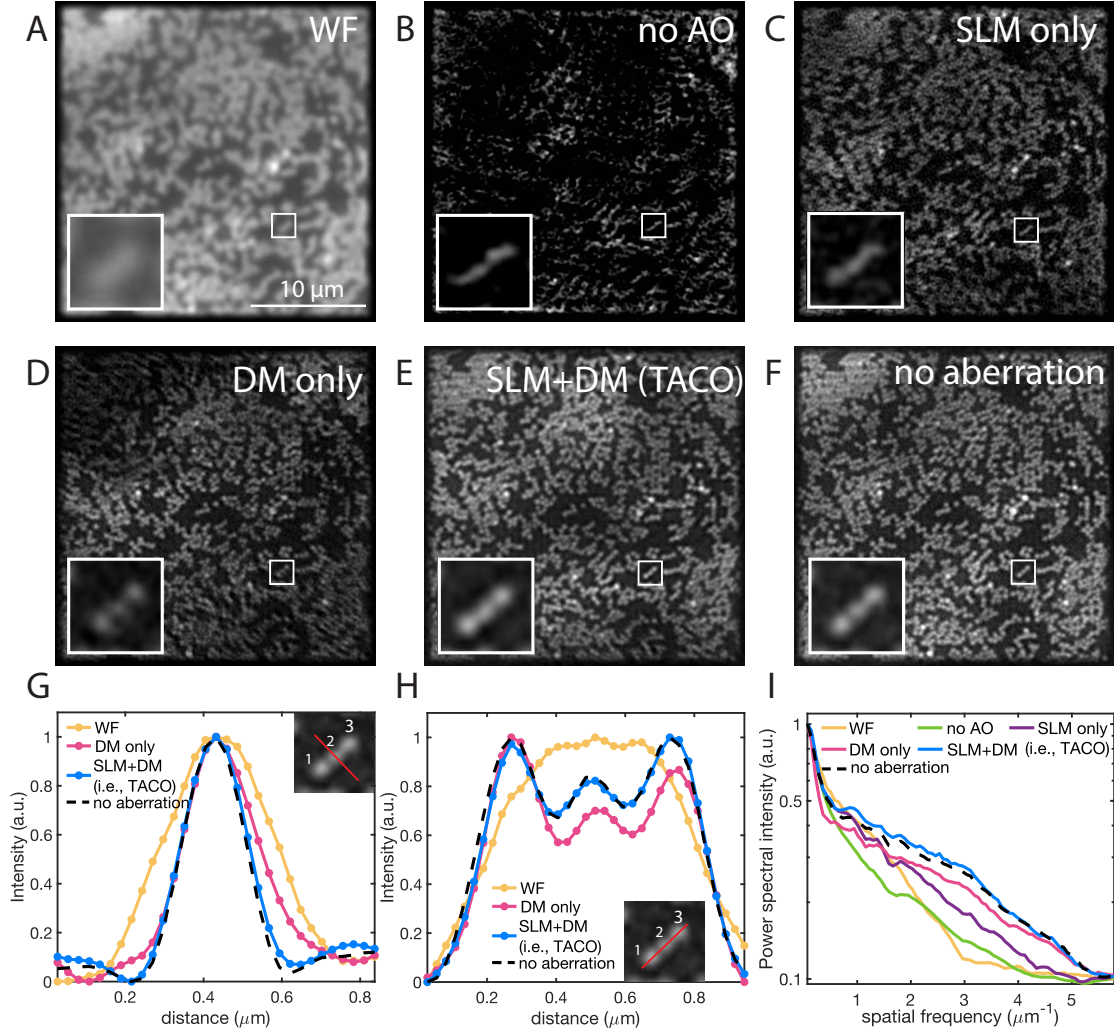


Figure 3.12: **Experimental SIM imaging of 200 nm diameter fluorescent bead sample.** (A) Wide-field image of the fluorescent bead sample. (B-F) SIM reconstructed images of the fluorescent bead sample for different aberration correction conditions. (B) No AO correction was performed. (C) Only SLM correction was employed. (D) Only DM correction was employed. (E) Both SLM and DM correction (i.e., TACO) were employed. (F) No aberration was introduced. (G) Normalized intensity profiles along the diagonal cut through bead 2 as shown by the red line in the inset. The profiles of all 3 beads are shown in Fig S7. FWHM values of beads 1-3 are: WF, 343 nm, 331 nm, 327 nm; DM only, 234 nm, 221 nm, 224 nm; SLM+DM, 207 nm, 191 nm, 183nm; no aberration, 190nm, 182 nm, 188nm. (H) Normalized intensity profiles along the diagonal cut through the 3 beads shown by the red line in the inset. The measured distances between the three peaks are: left, 216 nm; right, 243 nm. (I) Power spectrum of images for different conditions. Each curve is normalized by its intensity at zero frequency. Scale bar in A: 10 μm . Effective pixel size: 27 nm.

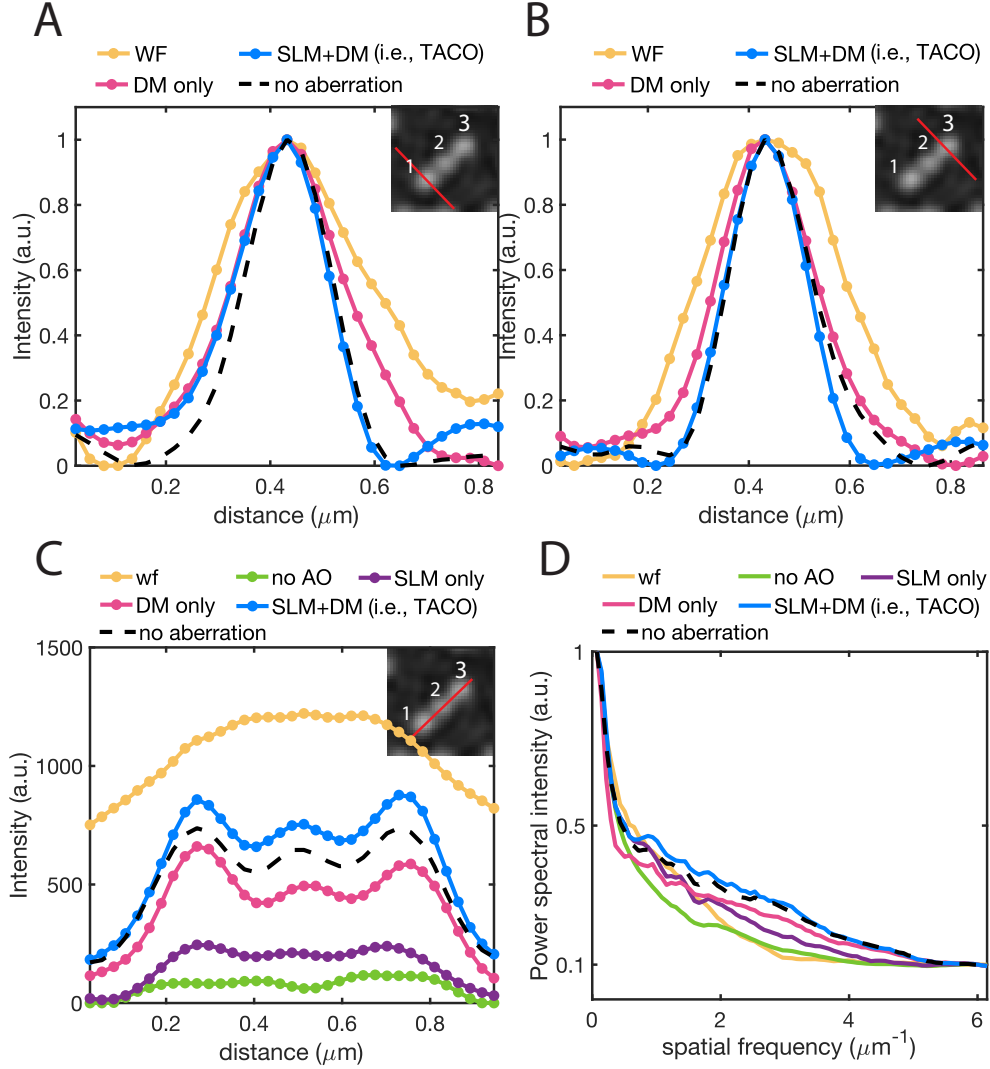


Figure 3.13: **Intensity profiles of fluorescent beads for different imaging conditions.** (A) Normalized intensity profile of bead 1 along the red line as shown in the inset. (B) Normalized intensity profile of bead 2 along the red line as shown in the inset. (C) Unnormalized intensity profile of the 3 adjacent beads along the red line as shown in the inset. The TACO correction shows an even higher signal level compared with no aberration because of the non-uniformity of illumination intensity that arises as a byproduct of the SLM phase modulation. Since the SLM phase modulation cannot completely cancel out the aberration, the final illumination wavefront after SLM correction still deviates slightly from an ideal plane wave, and the intensity distribution can be non-uniform due to the existence of residual phase modulation. (D) Power spectrum of the same analysis is shown in Fig. 3.12I with a linear scale y-axis instead of a log scale.

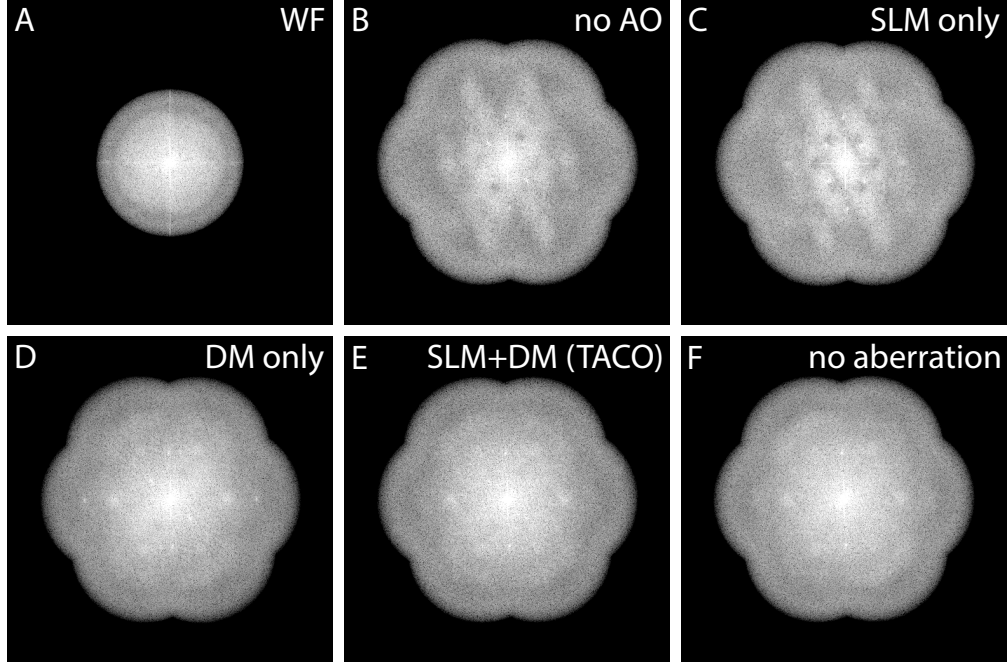


Figure 3.14: **Fourier transforms of the wide-field (WF) and structured illumination microscopy (SIM) reconstruction images** shown in Fig. 6. In the case of no AO (B) and SLM only (C), the FTs show obvious sub-structures that correspond to the strong global artifacts in the (corresponding) SIM reconstructions. These sub-structures are almost completely absent in the FT of the DM-only (D), TACO (E), and no aberration cases (F), because the global aberrations have been largely corrected by the DM. However, strong local aberrations persist in the DM-only case that can be clearly observed in the Fig. 6D.

shows a clear difference between the different reconstruction strategies. Although DM-only correction shows a much sharper profile (FWHM = 221 nm) compared to WF (FWHM = 331 nm), it is still significantly worse than the no aberration case (FWHM = 182 nm). This can also be seen in the distorted shape of the bead image. On the other hand, TACO correction leads to a further improved bead profile (FWHM = 191 nm) that is very close to the ideal case. Similar results can be seen from the measurements of beads 1 and 3 (Fig. S8A&B). The reason that the FWHM is less than 200 nm might be due to the variance of the manufacturing process. Fig. 3.12H further shows the profiles of the three adjacent beads in the insets. Although the DM-only correction is good enough to show distinguishable profiles of the 3 beads, the profiles of TACO correction manifest greater fidelity, similar to the unaberrated ideal case. Finally, the power spectra of the images for different imaging and correction conditions are obtained from their FTs (Fig. S9) and shown in Figure 3.12I. The y-axis was set to have a log scale to better illustrate the differences between each curve. We also show a linear scale version of the power spectrum analysis in Figure S8D. Compared with the wide-field condition, all the other images show an expansion of the distribution to higher spatial frequency. However, in the case of no AO correction or SLM-only correction, the intensity of high-frequency components is very limited. The case of DM-only correction shows improvement in the high-frequency range but is still inferior to the no-aberration case. By contrast, full implementation of TACO (i.e., both DM and SLM) enhances the frequency distribution to almost the same power spectrum as the ideal case. This further illustrates that the overall quality of the SIM reconstruction after implementing TACO is close to optimum.

3.4.3 *TACO-SIM imaging of biological samples*

We further tested TACO aberration correction on two different types of cell samples, human fibroblast cells, and human melanoma cells. We labeled the actin networks of the cells with

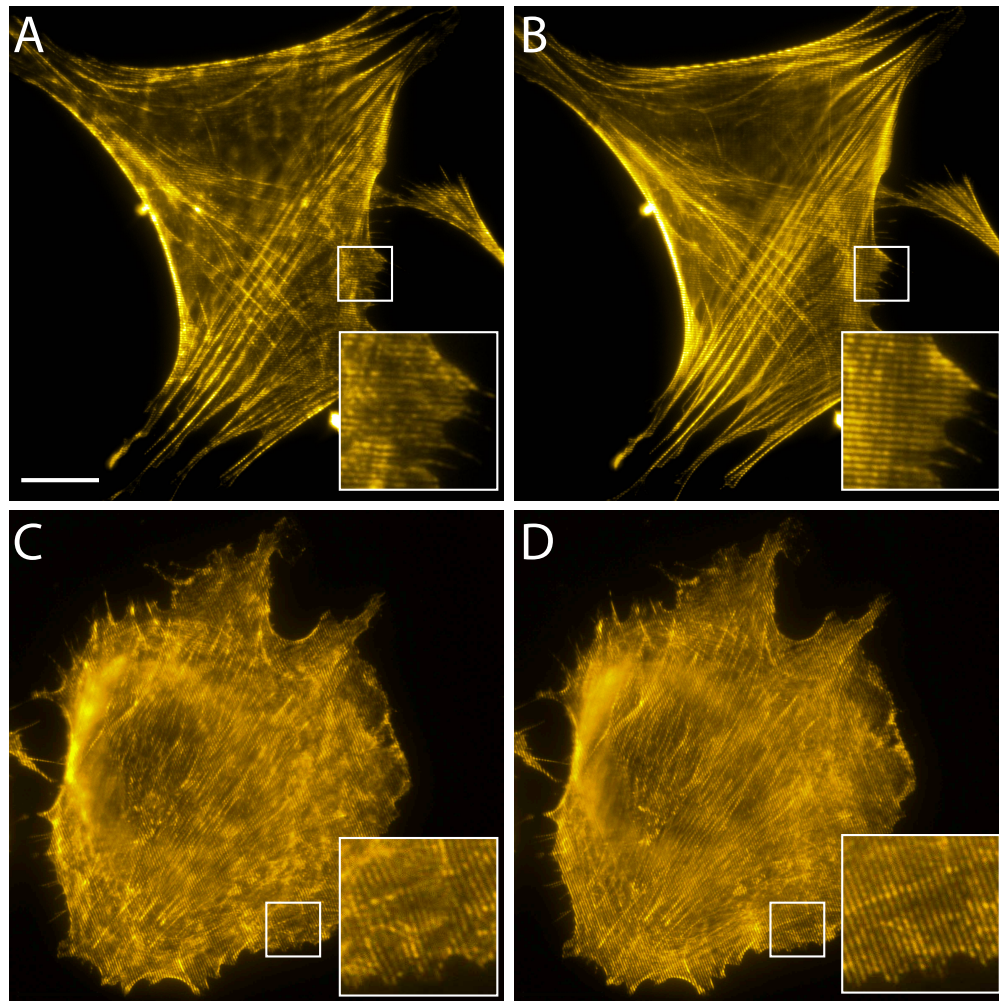


Figure 3.15: **Cell images obtained using sinusoidally modulated structured illumination (SIM) patterns before and after TACO.** Before TACO, the strong aberration from the optical aberration slide severely distorted the illumination pattern in both the human fibroblast cell (A) and the human melanoma cell (C). This distortion led to poor SIM reconstruction with significant artifacts (Fig. 3.16B&E). After the aberration was corrected by TACO, the illumination pattern was successfully restored (B, D). These high-quality SIM images and their $\pm 60^\circ$ oriented complements allow high-fidelity SIM super-resolution reconstructions (Fig. 3.16C&F). Scale bar in A: 20 μm .

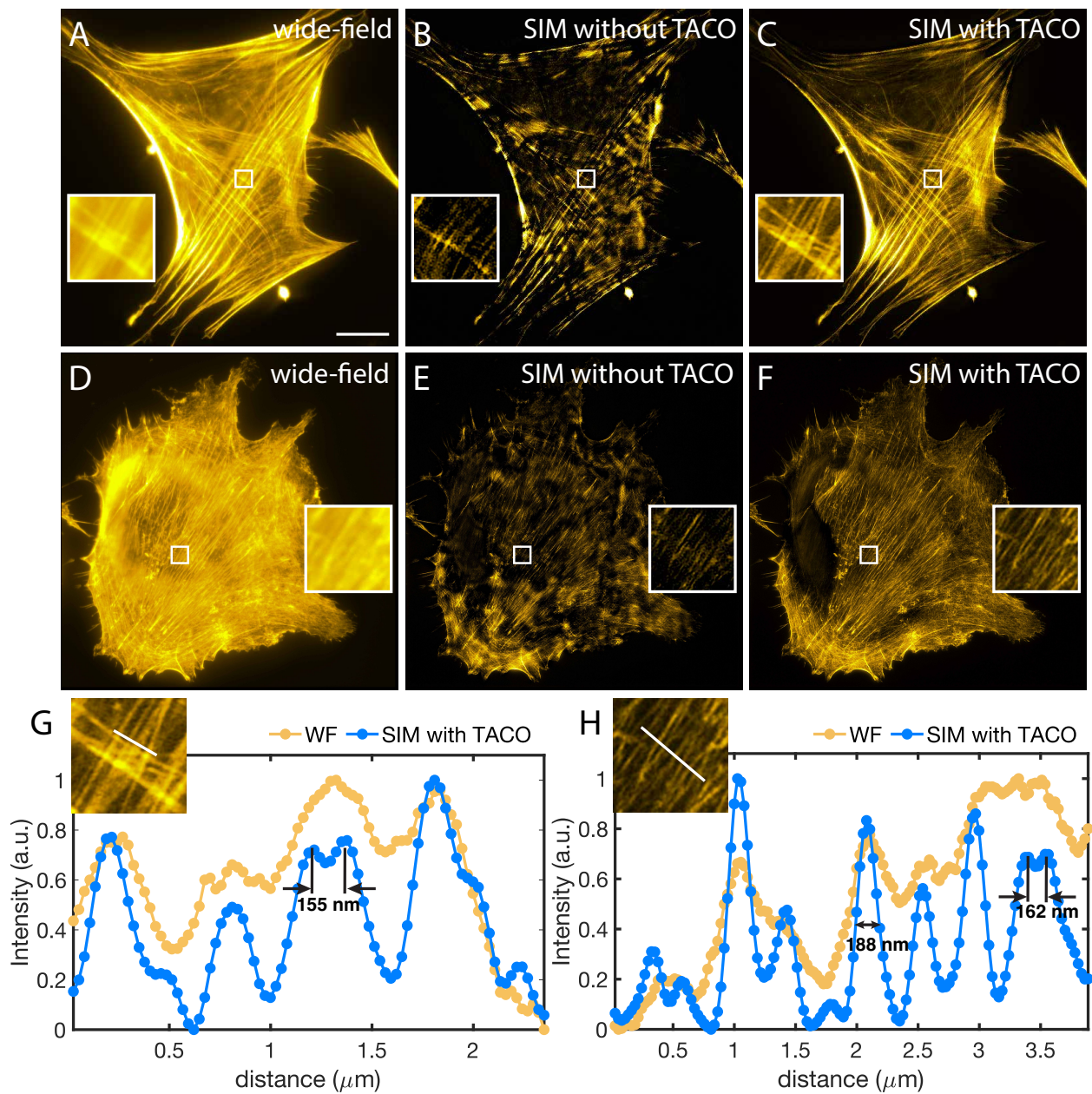


Figure 3.16: **Demonstration of TACO to imaging of phalloidin labeled actin in a human fibroblast cell (upper row) and a human melanoma cell (lower row).** (A, D) Wide field images. (B, E) SIM reconstruction without TACO. (C, F) SIM reconstruction with TACO. No additional brightness/contrast adjustment has been added to the images. (H, I) Normalized intensity profile along the lines as shown in the insets. Scale bar in A: 20 μm . Effective pixel size: 27 nm.

Alexa Fluor 555 phalloidin fluorescent dye and performed SIM imaging with and without the aberration slide. Wide-field imaging without any introduced aberration was first conducted (Fig. 3.16A &D). The images show poorly-resolved actin structures and strong out-of-focus backgrounds. Figure 3.16B &E show the reconstruction of SIM imaging, including aberration, but without TACO. Due to the strong perturbation from the aberration slide, the SIM pattern was severely distorted (Figure 3.15A &C) and cannot be reconstructed into accurate super-resolution images. Therefore, one should have low confidence in the cellular features obtained due to the potential artifacts associated with the aberrated illumination patterns. By contrast, the aberration was corrected and the sinusoidal pattern was successfully recovered using TACO (Figure 3.15B &D), which is what high-quality SIM reconstruction requires. As seen in Fig. 3.16C &F, SIM reconstructions after TACO show significantly improved quality, with the artifacts largely eliminated. Compared with wide-field images, the SIM reconstructed super-resolution images show much more detail of the actin networks with improved resolution and contrast and suppression of the out-of-focus light. As shown in 3.16G &H, the profile of the actin filaments is significantly more defined after SIM reconstruction with TACO correction, and previously indistinguishable actin structures, such as actin branching with separations as small as 155 nm, can be readily resolved.

3.5 Discussion and Conclusions

In general, research and application of adaptive optics in SIM have almost exclusively focused on the pupil AO configuration (Arigovindan et al. (2012); Débarre et al. (2008); Paudel et al. (2015); Thomas et al. (2015); Turcotte et al. (2019); Li et al. (2020)). However, unlike point-scanning microscopy, wide-field illumination is quite immune to global aberrations like spherical aberration or coma Booth et al. (2015); Turcotte et al. (2019), but is highly susceptible to spatially-varying aberrations, e.g., caused by sample inhomogeneity or non-flatness of optical components such as DMDs (Brown et al. (2021)). Consequently, the

conventional pupil AO aberration correction approach, which is suitable for the correction of global aberrations but is not able to correct spatially-varying aberrations, is not sufficient for wide-field microscopy techniques such as SIM. In this work, we demonstrated a tandem aberration correction optics - TACO - method to correct both global and local aberrations in SIM.

The present version of TACO incorporates a DM for pupil AO in the optical fluorescence detection path to correct for global aberrations, while an SLM is used for conjugate AO in the optical illumination path to compensate for locally-varying aberrations. We demonstrate that this TACO system is capable of restoring severe SIM pattern distortion, and enables high-quality super-resolution reconstruction that cannot be recovered by pupil AO alone. We note that the main focus of this work is to demonstrate a solution for the long-neglected local aberration lurking in the SIM illumination, instead of the well-studied (both local and global) aberrations in the emission path. Thus the current setup has the conjugate AO only functioning in the illumination path to correct for the spatially-varying aberration caused by the optical aberration slide, while for the emission path a basic pupil AO method is adopted. This optical setup can be expanded to allow imaging of thick aberrating samples by adding a conjugate AO corrector in the detection path to further correct for the spatially-variant aberrations in the detection path (Li et al. (2015)). Multi-conjugate AO (Bedggood et al. (2006); Simmonds and Booth (2013); Wu and Cui (2015)), or refocusing conjugate AO (Tao et al. (2017)) can also be adopted to accommodate a more realistic aberration sample, such as thick biological tissues.

One limitation of the current setup is that the position of the SLM, near the image plane, leads to the projection of its pixelated image onto the sample plane, causing artifacts in the SIM reconstruction. One solution is to use a wavefront modulator with a more continuous wave shaping function, such as a deformable mirror, or an SLM device with a 100% fill factor (e.g. Meadowlark Optics SLM) that does not show observable pixelation. The present

experimental advance (TACO) can also be complemented by computational post-processing. Recently, computational adaptive optics has been shown to compensate aberrations based on purely computational models without the need for wavefront corrector hardware (Adie et al. (2012); Kwon et al. (2023)), providing much more flexibility in the choice of the conjugate plane and AO process. This post-processing approach would complement our hardware-based TACO method.

While we have demonstrated the effectiveness of the TACO approach, we acknowledge that in practical applications, efforts to accelerate the AO correction speed are crucial to enhance the imaging process. For instance, more efficient algorithms like the multidither coherent optical adaptive technique can be employed, which divides the wavefront corrector into independent segments and determines the phase of each segment in parallel (Cui (2011); Zhao et al. (2019); Liu et al. (2014)). Novel devices for direct wavefront sensing have also been designed for large fields of view, such as partitioned aperture wavefront (PAW) sensor (Li et al. (2015)) and spinning-pupil aberration measurement (SPAM) device (Furieri et al. (2022)), which can be used to measure wavefront aberration directly and eliminate the need to do iteration on an image metric.

Overall, the TACO method demonstrates the ability to maintain high-quality SIM illumination and reconstruction when imaging through highly aberrating optical media. Furthermore, The TACO method is not limited to SIM and can also be applied to other novel wide-field microscopy techniques, such as multi-color DMD-SIM (Gong et al. (2024)), image scanning microscopy (Müller and Enderlein (2010); York et al. (2012)), multi-plane microscopy (Xiao et al. (2020); Descoux et al. (2020)) and temporal focusing microscopy (Oron et al. (2005)). Furthermore, TACO can be used to correct non-uniformity of the illumination even for conventional wide-field microscopy. Therefore, this method fills a long-standing gap in applying the AO technique to wide-field-based illumination.

TACO should improve wide-field imaging techniques in biological research, especially for

imaging through thick tissues, such as brains or embryos, which can cause strong aberration of the illumination light. The TACO system we demonstrate can greatly mitigate this problem and make possible high-quality wide-field illumination patterns across highly scattering tissues. Compared with conventional point scanning microscopy such as confocal and 2-photon, wide-field imaging techniques have the advantage of being able to image the entire FOV at once without the need to scan over the FOV in a time series, and thus could significantly increase the imaging frame rate thereby revealing more details of biological dynamics that were previously inaccessible.

Appendix 3.A SPGD

3.A.1 Principle of SPGD

In the present work, we used the SPGD algorithm Vorontsov and Sivokon (1998) for the iteration and the step-wise increase of the image quality metric for both the SLM (metric defined in Equation 2) and deformable mirror (DM) where we use image contrast as metric. We give a brief introduction to the algorithm, but more details of the algorithm are presented in Vorontsov and Sivokon (1998). Consider an image quality metric, M , that is a function of the phase modulation of each controllable segment of a wavefront corrector, $\phi = \{\phi_1, \phi_2, \dots, \phi_N\}$ so that

$$M(\phi) = M(\{\phi_1, \phi_2, \dots, \phi_N\}) \quad (3.3)$$

When small perturbations $\{\delta\phi_1, \delta\phi_2, \dots, \delta\phi_N\}$ are applied to the phase modulation device, the change of the metric M can be expressed as the Taylor series expansion:

$$\delta M = \sum_{i=1}^N \frac{\partial M}{\partial \phi_i} \delta \phi_i + \frac{1}{2} \sum_{i=1}^N \sum_{j=1}^N \frac{\partial^2 M}{\partial \phi_i \partial \phi_j} \delta \phi_i \delta \phi_j + \dots \quad (3.4)$$

Consider the phase modulation ϕ_k from a specific segment k . Multiplying $\delta\phi_k$ to both sides of equation 3.4 gives

$$\delta M \delta\phi_k = \frac{\partial M}{\partial\phi_k} (\delta\phi_k)^2 + n_k \quad (3.5)$$

in which n_k is the noise term

$$n_k = \sum_{i \neq k}^N \frac{\partial M}{\partial\phi_i} \delta\phi_i \phi_k + \frac{1}{2} \sum_{i=1}^N \sum_{j=1}^N \frac{\partial^2 M}{\partial\phi_i \partial\phi_j} \delta\phi_i \delta\phi_j \delta\phi_k + \dots \quad (3.6)$$

The phase perturbations are chosen to be statistically independent random variables that have zero means $\langle\phi_i\rangle = 0$ and equal variances $\langle\phi_i\phi_j\rangle = \sigma^2\delta_{ij}$, in which $\delta_{ij} = 1$ when $i = j$ and $\delta_{ij} = 0$ when $i \neq j$. Then the expectation value of the first and second terms are all zero because $\langle\phi_i\phi_k\rangle = 0$ for all $i \neq k$ and $\langle\phi_i\phi_j\phi_k\rangle = 0$ for all i, j, k . In this way, the expectation value of the noise term is of the order of σ^4 , and can be expressed as $\langle n_k \rangle = \mathcal{O}(\sigma^4)$, and the expectation value of equation 3.5 can be expressed as

$$\langle \delta M \delta\phi_k \rangle = \frac{\partial M}{\partial\phi_k} \sigma^2 + \mathcal{O}(\sigma^4) \quad (3.7)$$

and an approximation for the gradient $\frac{\partial M}{\partial\phi_k}$ is

$$\frac{\partial M}{\partial\phi_k} = \frac{\langle \delta M \delta\phi_k \rangle}{\sigma^2} - \mathcal{O}(\sigma^2). \quad (3.8)$$

We can make the same approximation of the gradients for all the segments, and in this way, we can get approximations of the gradient components of metric M versus all segment values ϕ in parallel with an accuracy of $\mathcal{O}(\sigma^2)$. We can thus develop the iteration of the SPGD procedure as:

$$\phi_i^{m+1} = \phi_i^m - b \left(\frac{\partial M}{\partial\phi_i} \right) = \phi_i^m - b \delta M^m \delta\phi_i^m, \quad (3.9)$$

where m is the iteration step, and b is a constant to control the rate of each iteration. b is positive when the metric needs to be minimized and is negative when the metric needs to be maximized. In the present work, we used a variation of the standard SPGD algorithm by doing two measurements with 2 opposite perturbations in each iteration step so we get

$$\delta M_+ = M(\phi + \delta\phi) - M(\phi), \quad \delta M_- = M(\phi - \delta\phi) - M(\phi) \quad (3.10)$$

Therefore, for each iteration step, we update the phase values by

$$\phi_i^{m+1} = \phi_i^m - 2b(\delta M_+^m - \delta M_-^m)\delta\phi_i^m. \quad (3.11)$$

3.A.2 Realization of SPGD

In practice, an interactive Labview-based software interface was created to show the time-developing plot of metric value M and the images taken in each iteration in real time. The user can empirically determine the step size of the optimization and when to stop the optimization or switch to a different pattern and continue the optimization. The value of the step sizes, b , (for conjugate AO and pupil AO) are determined empirically by trying different step values and monitoring the resulting trends of the metric value. The values are chosen to balance the speed of convergence against the risk of excessive oscillations. The values can be determined reasonably quickly within one optimization cycle, and once they are determined they remain effective for similar types of samples, minimizing the need for further adjustment. In our case, we have different definitions of metric values: for pupil AO, the metric value is generally large, so it leads to a relatively small step size; and for conjugate AO, the metric value is generally small, so the corresponding step size is relatively large. Specifically, the step size b for pupil AO was 1-5 with the perturbation size $\delta\phi_i$ of each Zernike coefficient set to be 0.001, and the step size b for conjugate AO was 100-500 with the perturbation size $\delta\phi_i$ of

each SLM pixel value set to be 10. The process could be sped up by having an adaptive value, but for the present purpose we were satisfied with the more straightforward approach. The convergence of an optimization cycle was determined by visual inspection of the metric value reaching a plateau. The simplicity and clarity of the curve shapes make the determination of the convergence very easy, straightforward, and reliable. Any inconsistency arising from the operator's judgment does not significantly impact the overall optimization process. It's also important to highlight the flexibility and adaptability offered by including operator judgment in our experiment, particularly when dealing with unexpected variations, such as random external disturbances that might include vibrations or electronic instabilities. The operator's ability to respond to these external fluctuations ensures a more robust optimization process compared to rigid, predefined criteria. Specifically, for Figure 3.11, the different patterns were executed for sufficient amounts of time to determine a plateau behavior for each. Finally, in that figure we return to pattern 1 to verify whether the other pattern optimizations also affected pattern 1.

Appendix 3.B TACO control

3.B.1 pupil AO in TACO

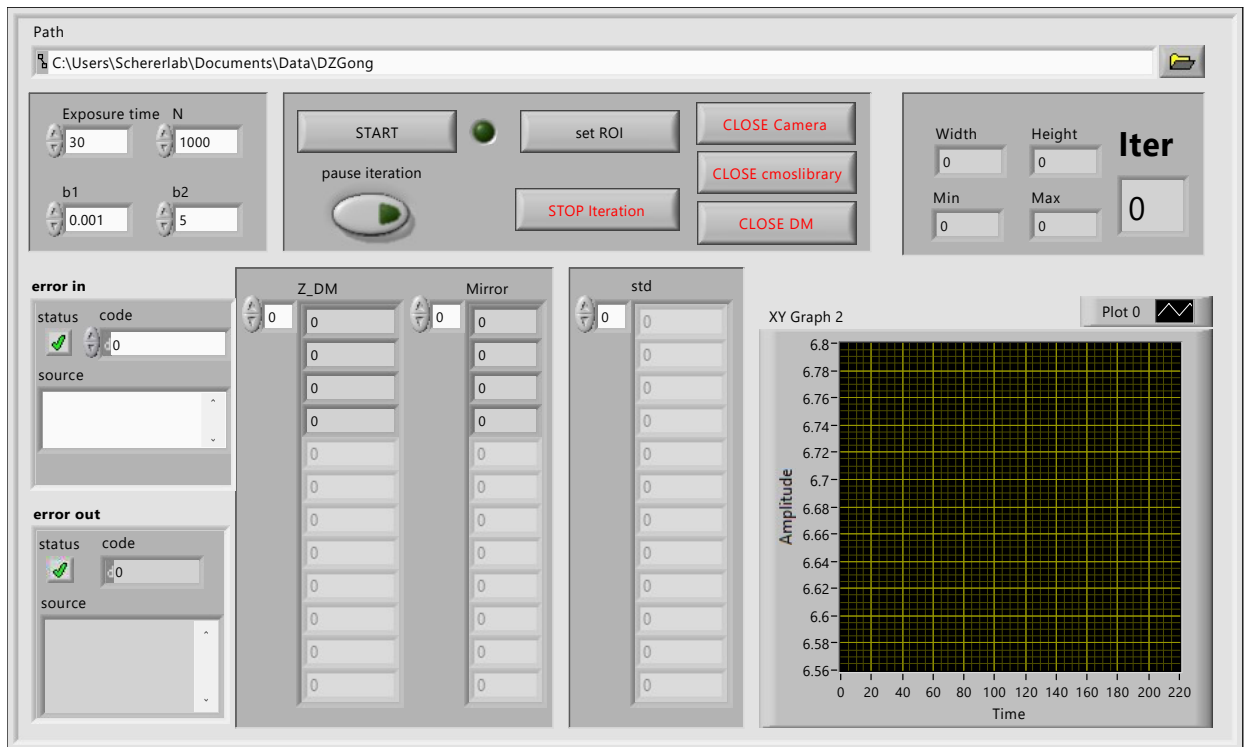


Figure 3.17: Front panel of DM pupil AO control

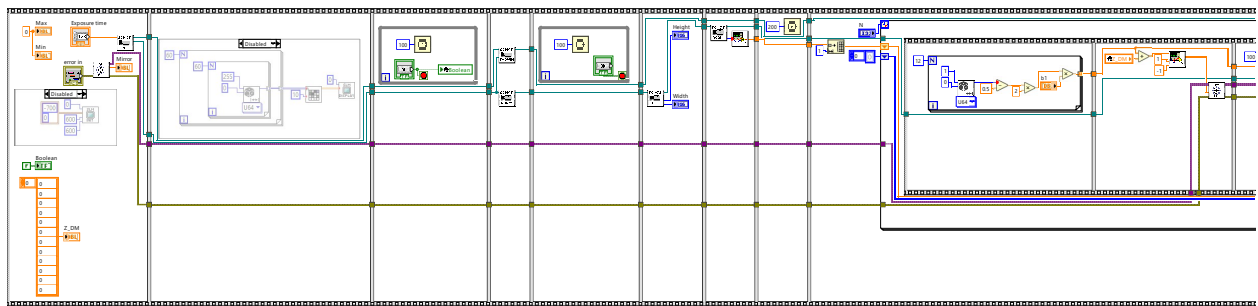


Figure 3.18: Block diagram of DM pupil AO control part 1

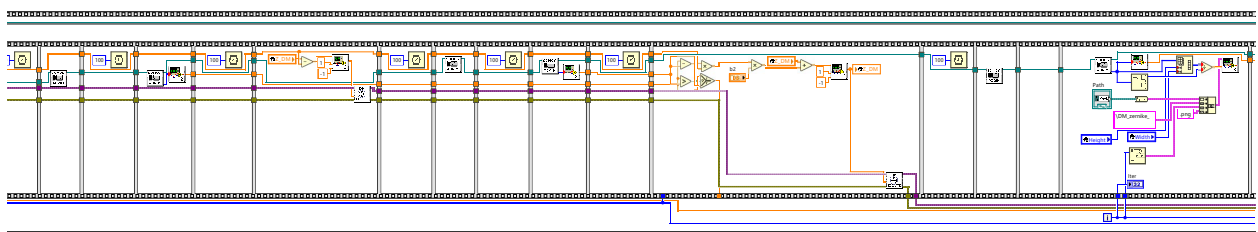


Figure 3.19: Block diagram of DM pupil AO control part 2

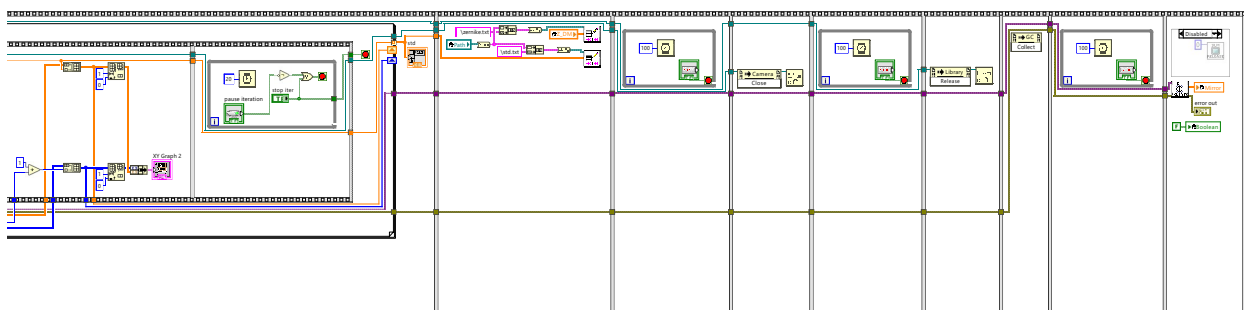


Figure 3.20: Block diagram of DM pupil AO control part 3

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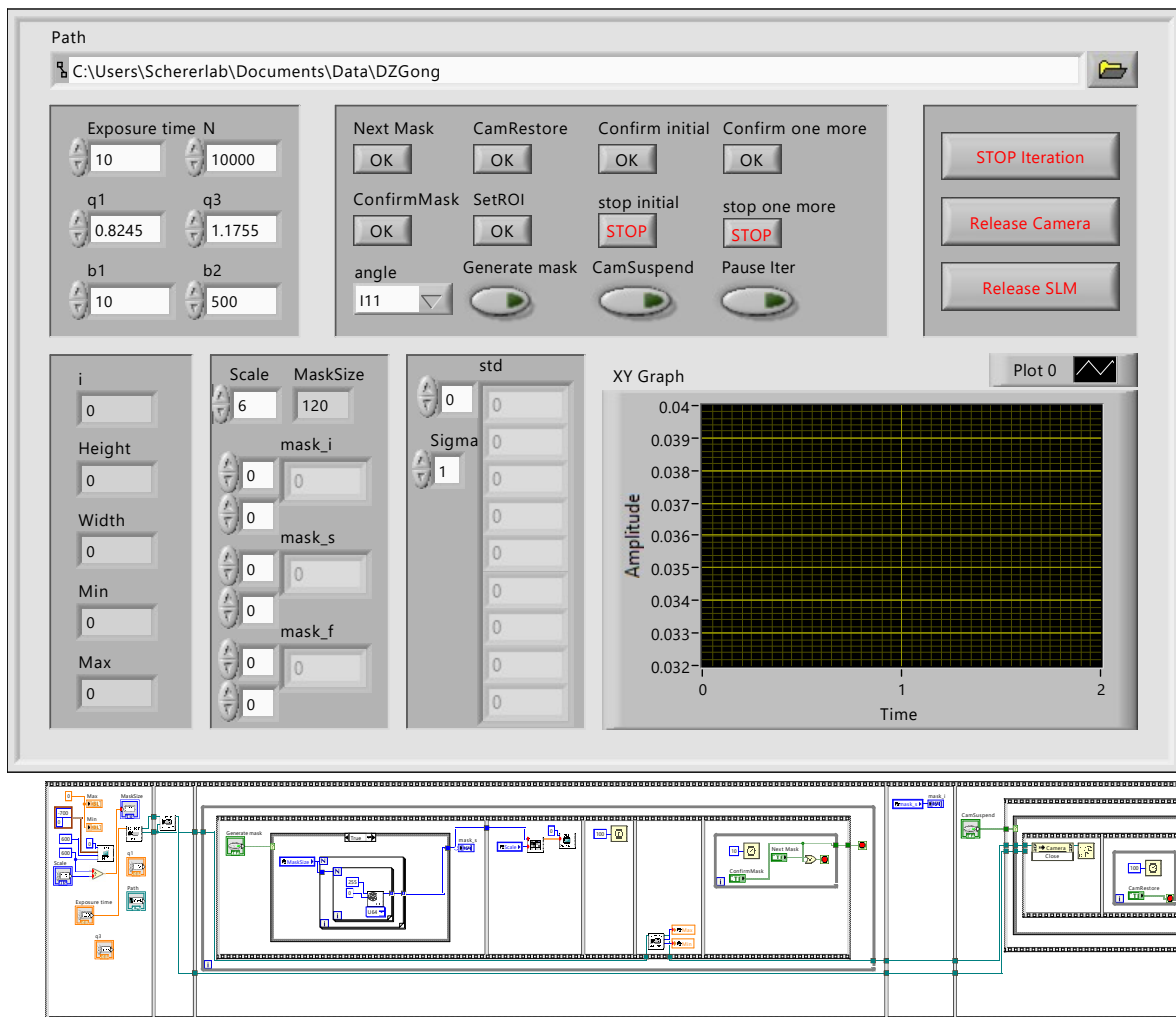


Figure 3.21: Labview code: CMOS_SLM_RandomMatrix part1

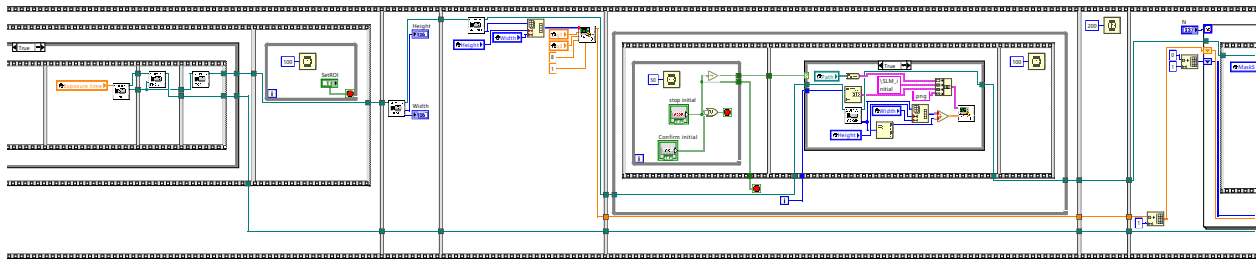


Figure 3.22: Labview code: CMOS_SLM_RandomMatrix part2

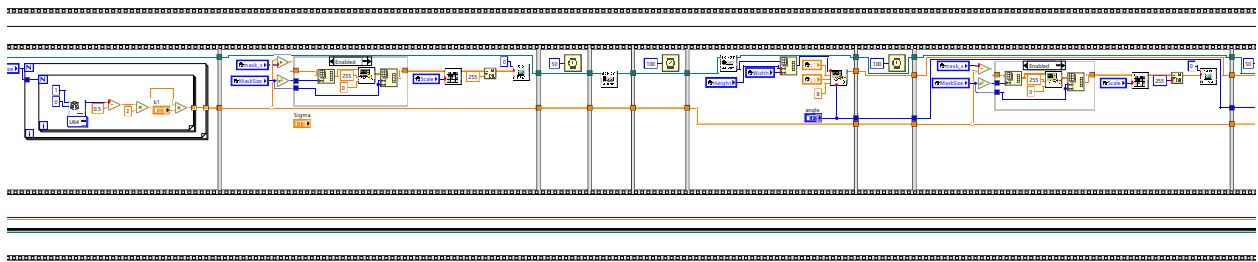


Figure 3.23: Labview code: CMOS_SLM_RandomMatrix part3

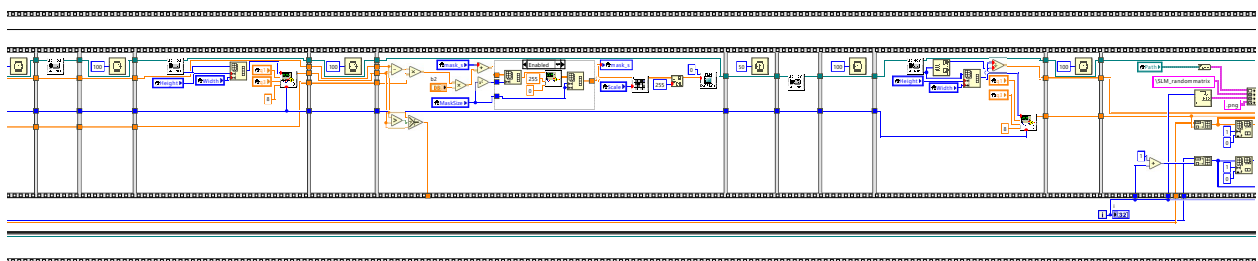


Figure 3.24: Labview code: CMOS_SLM_RandomMatrix part4

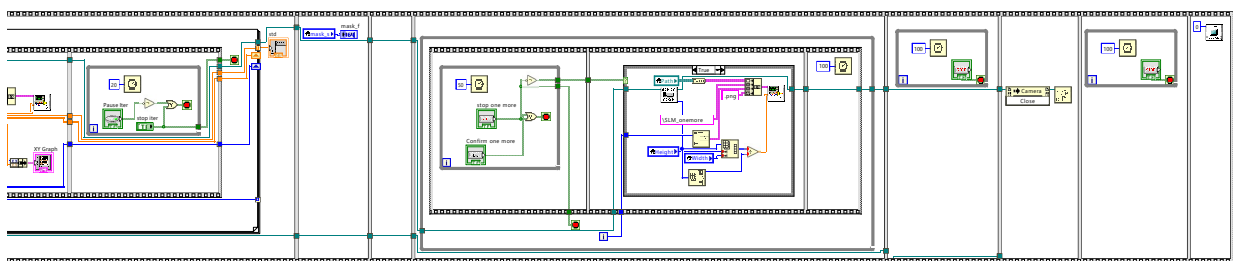


Figure 3.25: Labview code: CMOS_SLM_RandomMatrix part1

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CHAPTER 4

MS-SIM: MULTI-SCAN FAST 3D-SIM

4.1 Introduction

The resolution of conventional optical microscopy is inherently constrained in both lateral and axial dimensions. This resolution limitation is known as the diffraction limit (Pawley (2006)). Over the past few decades, various super-resolution imaging techniques have been developed to transcend this barrier (Schermerle et al. (2019); Bond et al. (2022); Liu et al. (2018)). Among these, Structured Illumination Microscopy (SIM) offers a modest ($\sim 2\times$) resolution enhancement compared to techniques capable of achieving single molecule imaging and nanoscale localization. However, SIM boasts significant advantages, including the need for fewer frames for image reconstruction compared to other super-resolution techniques, lower excitation intensity, and no restrictions on the choice of labels (Zhao et al. (2021); Chen et al. (2023)). These attributes position SIM as a technique exceptionally suitable for live cell imaging.

Standard SIM utilizes sinusoidal illumination patterns, typically generated by the interference of 2 coherent beams, to down-modulate high spatial frequency information into beat patterns with lower frequencies that can be resolved by the objective (Gustafsson (2000)). Combining the high spatial frequency information of the sample extracted from a series of images acquired with (striped) patterns at 3 or more different orientations and phases allows for the reconstruction of a super-resolution image with theoretically up to $2\times$ improvement in lateral resolution. However, the patterns generated from 2-beam-interference are invariant along the axial direction. Therefore, they cannot provide axial resolution enhancement or optical-sectioning. As a result, 2-beam-interference SIM suffers from out-of-focus light, and its application in 3D imaging is limited (Gustafsson et al. (2008)). Although post-processing-based methods have been developed to obtain optical sectioning, they sacrifice some of the

lateral resolution enhancement (O’Holleran and Shaw (2014)).

In contrast, 3D-SIM, which does not compromise lateral resolution, can be achieved by utilizing 3-beam-interference, which provides modulation in both the lateral and axial directions. The reconstruction of a whole z-stack of axially sectioned SIM images can lead to resolution enhancement by up to $2\times$ in all three dimensions (Gustafsson et al. (2008)). However, reconstruction of a 3D-SIM image requires an image series with 15 different patterns (3 orientations; 5 phase shifted patterns) at each z position. This lengthy process of image acquisition dramatically slows volumetric imaging speed and exacerbates issues of photobleaching and phototoxicity, significantly obstructing the application of 3D-SIM in live cell imaging.

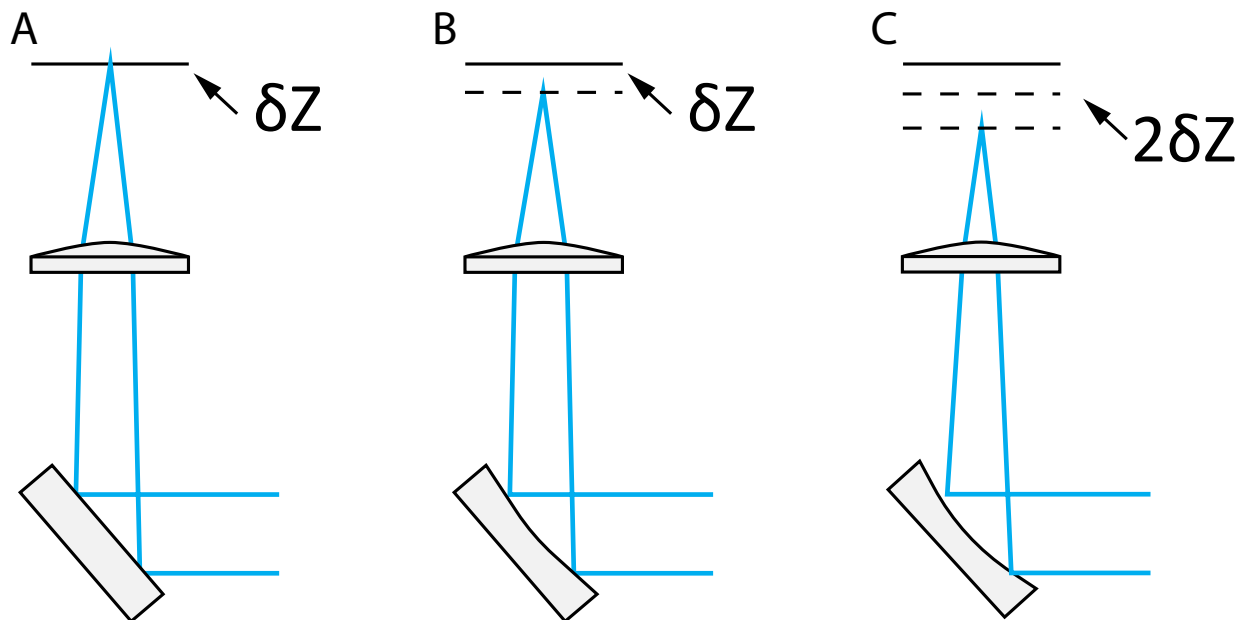


Figure 4.1: **Remote focusing by deformable mirror.** Inertia-free focal shift can be achieved by adjusting the surface curvature of the deformable mirror. For instance, deformable mirror can rapidly change the focal plane by deforming its surface to different curvatures, as shown in A-C.

One strategy to accelerate the 3D-SIM image acquisition is to perform fast z-scanning by employing remote focusing using optical elements that can rapidly change focal length (hence focal plane in the sample), such as tunable-focus lenses (Hinsdale et al. (2015)) or deformable

mirrors (Stoychev et al. (2023)). Compared to moving the objective or sample stage along the z-axis, this nearly inertia-free method is much more rapid and stable. Furthermore, update rates of approximately kHz minimize the dead time between consecutive z-scans (Fig. 4.1). However, the sequential imaging with 15 patterns at each z-position still takes a significant amount of time.

Another strategy that can significantly reduce the exposure time of sequential imaging in 3D samples is to simultaneously image multiple focal planes by multiplexing the fluorescence signal from n different focal planes (Fig. 4.2). This approach shortens the image acquisition time for the whole volume n fold. Both diffractive methods, such as using specially manufactured diffractive optical elements (Abrahamsson et al. (2017); Senftleben et al. (2024)), and refractive methods, such as using specially designed prisms (Descoux et al. (2020)) or combinations of multiple beamsplitters (Johnson et al. (2022)), have been applied in SIM to achieve high-speed imaging. However, fluorescence-splitting also leads to a decrease in signal level by n times at each focal plane. Achieving acceptable SNR in a reasonable amount of time necessitates limiting the maximal number of z-slices to be less than 10. Furthermore, the image aberration between different focal planes makes it difficult to conduct high-quality 3D-SIM reconstruction.

The novel multi-scan structured illumination microscopy (MS-SIM) scheme we introduce in this chapter combines both multiplexing and fast z-scanning, enabling fast 3D-SIM live cell imaging. We utilize a home-built multiplane image splitter Xiao et al. (2020) to focus the fluorescence signal from three axially separated positions to three adjacent areas of the detector, which allows simultaneously obtaining SIM images from three image planes simultaneously (Fig. 4.3a). Additionally, rapid remote focusing is achieved using a deformable mirror to axially scan these three image planes through three stacked volumes. This MS-SIM system enables 3D-SIM imaging through large (give 3D dimensions) volumes at high speed. We first calibrated our system using a fluorescent bead sample. We then conducted live cell

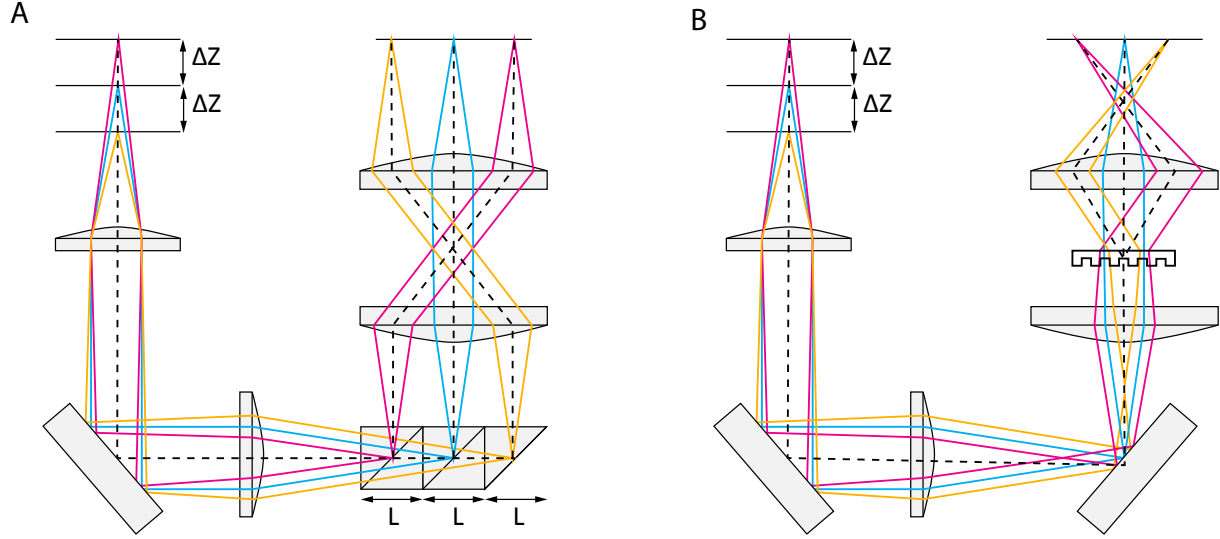


Figure 4.2: **Two configurations for snapshot volumetric imaging.** (A) Multiplane imaging using beam splitters to translationally split the detection path at the image plane. (B) Multifocal imaging using a diffractive optics element to angularly split the detection path at the pupil plane.

imaging, demonstrating that 3D-SIM volumetric imaging of a whole cell can be performed at speeds up to 1 Hz.

4.2 Experimental method

4.2.1 Experimental setup

Figures 4.3 and 4.4 illustrate the 2D and 3D schematics of the MS-SIM system, respectively. The illumination path remains consistent with the basic DMD-SIM setup described in Chapter 2. For the sake of clarity in the figures, optical elements not central to this Chapter, such as the SLM used for aberration correction in the Chapter 3 and the grating for multi-color imaging in the Chapter 5, have been omitted. However, in real experiments, both of these modules are mounted on kinematic bases, allowing for easy integration into the MS-SIM optical setup.

In the MS-SIM setup, the key components include the deformable mirror and the image

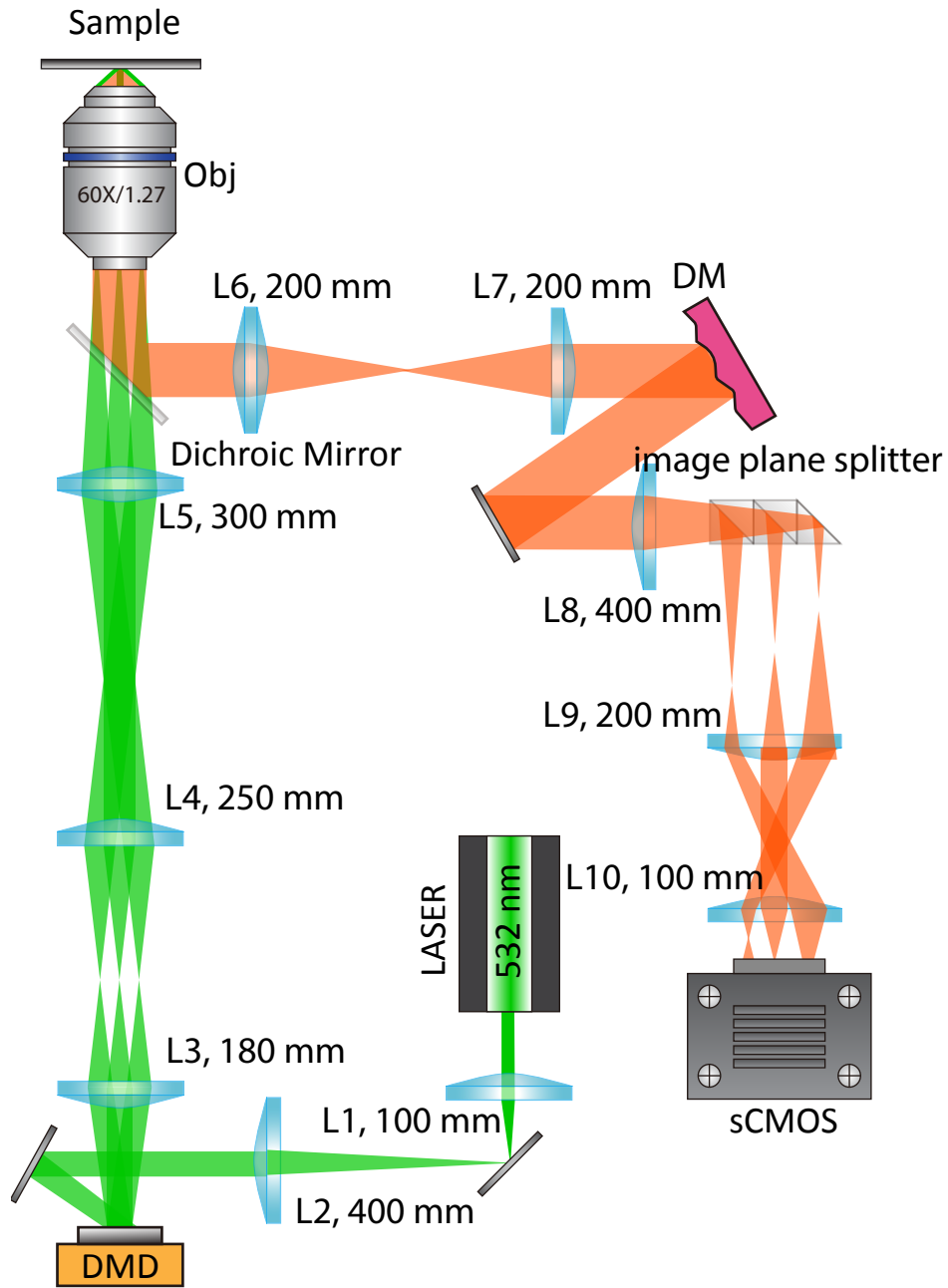


Figure 4.3: **2D schematic of the multi-scan SIM setup.** The detailed description of the setup is presented in the maintext.

plane splitter in the detection path to achieve remote focusing and multi-plane imaging. The deformable mirror (Thorlabs DMH40-P01) is placed at the pupil plane between a pair of lenses with 200/400 mm focal lengths. The control of the deformable mirror is identical to that shown in the Chapter 3. After the 400 mm lenses, the image plane splitter is employed to split the incoming fluorescence signal into three individual paths. Two types of image plane splitters have been constructed: one following the original design in Xiao et al. (2020), which uses a combination of a 30:70 non-polarizing beamsplitter (Thorlabs, BS052), a 50:50 non-polarizing beamsplitter (Thorlabs, BS004), and a right-angle prism (Thorlabs, PS614). A second version swaps the right-angle prism with a 90:10 non-polarizing beamsplitter (Thorlabs, BS073).

In both versions, the optical components are glued together using optical gel (Norland optical adhesive 65) and cured under UV light. There are several reasons for using a third 90:10 beamsplitter instead of a right-angle prism: firstly, in this design, all three optical components have nearly identical dimensions, ensuring that the intervals between each image plane are precisely the same. Secondly, the uniform dimensions and weights of the three components make it much easier to align them during gluing, ensuring that all edges are well aligned and have the same glue layer between each interface. Lastly, although the 90:10 beamsplitter transmits a small amount of signal, this portion of the signal is not necessarily discarded. In practice, an additional wavefront sensor can be used to collect this signal for wavefront sensing, and when paired with the deformable mirror, can achieve sensor-based aberration correction.

After the image plane splitter, a 200 mm/100 mm lens pair is used to relay the three sub-images to the sCMOS array detector (Photometrics, Kinetix). The reason for the demagnification is that the length of the image plane splitter is $12.5 \times 3 = 37.5$ mm, while the size of the sCMOS sensor is about 20 x 20 mm. The 2x demagnification ensures that the three sub-images can fit within the size of the camera.

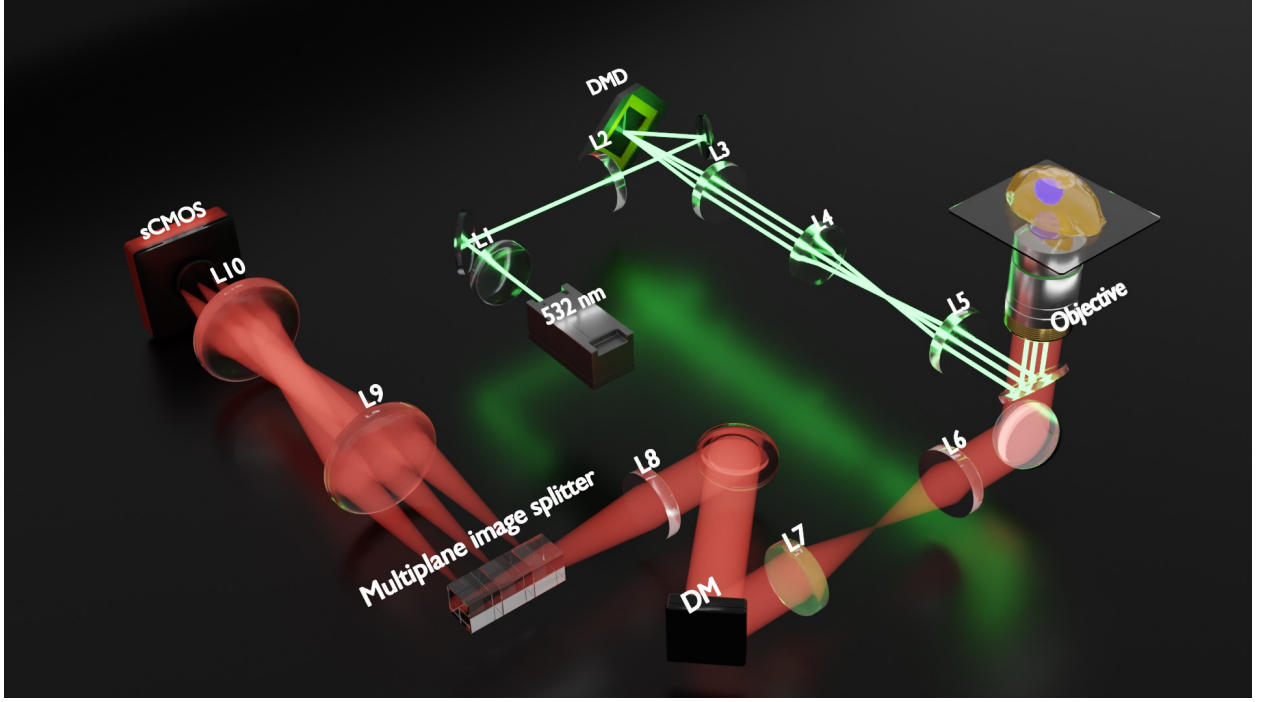


Figure 4.4: **3D schematic of the multi-scan SIM setup.** The layout and the detailed information of the hardware matches with the 2D schematic shown in Figure 4.3.

However, one issue with the multi-plane imaging design is that some of the sub-images are formed at the edges of the lenses, which can lead to aberrations, particularly field curvature. To mitigate this issue, instead of directly using a 200 mm and 100 mm lens pair, we utilize a combination of two 400 mm doublets and two 200 mm doublets to form the relay lenses. This configuration, also known as Plössl lenses, has been proven to effectively mitigate these aberrations (Tsang et al. (2021)).

The trade-off between lateral and axial field of view in single-camera snapshot volumetric imaging is a common caveat. In our setup, the formation of the three sub-images in a line means that each sub-image occupies less than $1/9$ of the total area of the image sensor, resulting in a reduction of the lateral field of view by $1/9$ compared to the maximum field of view.

In many scenarios, having a larger lateral field of view is more critical than simultaneously imaging three smaller fields of view in different axial directions. Therefore, it's highly

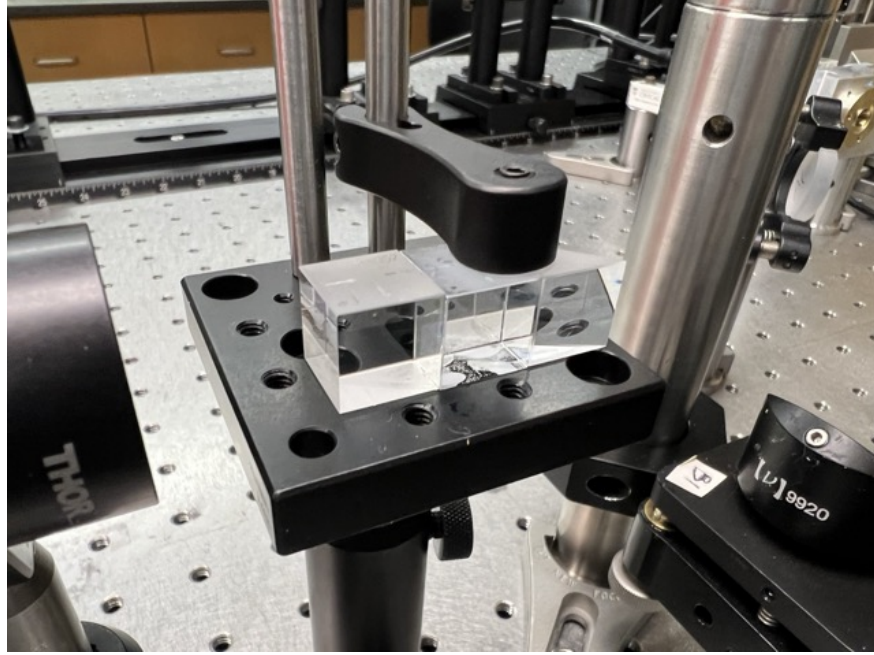


Figure 4.5: **Photo of the actual image plane splitter.** From the left to the right, the optics components are a 30:70 beamsplitter, a 50:50 beamsplitter and a right angle prism.

desirable to be able to conveniently switch between the conventional single plane large FOV imaging mode and the multi-plane imaging mode. To achieve this, the CMOS detector is mounted on a movable cage-system based track, and the image plane splitter is also mounted on a mechanical stage that can be moved out of the light path. When conventional large FOV 2D imaging is required, the camera can be moved to the focal plane of the 400 mm lens before the image plane splitter, and the image plane splitter can be moved out of the light path. This configuration ensures that the detection path remains the same as the TACO-SIM setup, allowing for large FOV 2D imaging without the constraints of multi-plane imaging.

4.3 Multi-scan imaging

4.3.1 *Calibration of multi-scan imaging*

The axial shifting caused by the defocus mode image plane separation of the image plane splitter is calibrated by axially moving a monolayer of fluorescent bead film sample using a

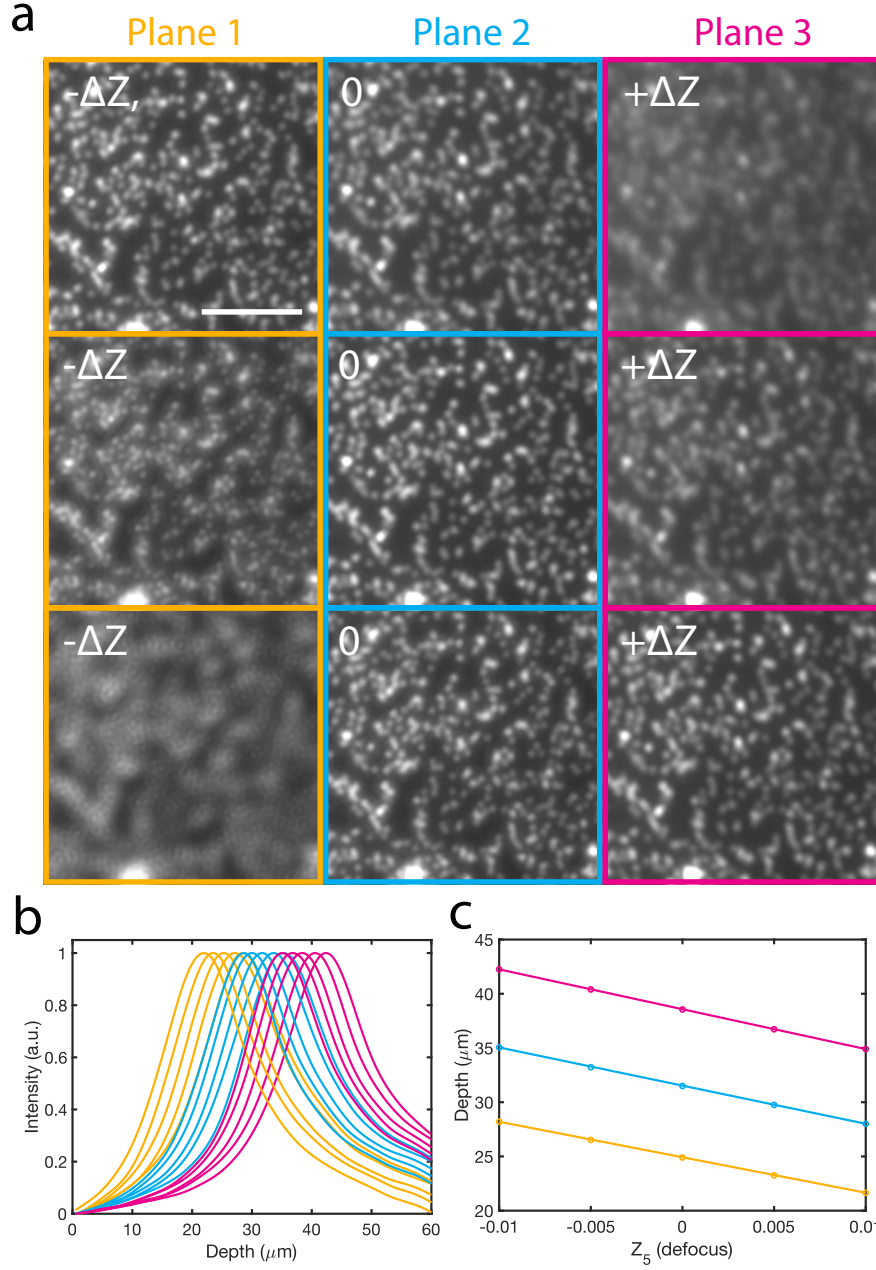


Figure 4.6: **Multi-scan wide field imaging of fluorescent beads.** (a) Wide field imaging of fluorescent beads at 3 different imaging planes 0, $\pm\Delta Z$ with different focal shifts created by deformable mirror in different rows. (b) Z-axis intensity profiles of the fluorescent beads imaged at different image plane and different values of the DM focal shift coefficient Z_5 . (c) Z-positions of the 3 image planes under different DM focal shift coefficient Z_5 values determined from the intensity profiles. Scale Bar: $5\mu\text{m}$

closed-loop piezo stage (MCL, piezo-z). In Figure 4.6, a series of values of the Z_5 Zernike polynomial, which corresponds to defocus (Noll (1976)), is applied to the deformable mirror. With each Z_5 value, a z-stack of the three image planes is captured by moving the piezo stage axially. The depth of each image plane and the defocus of the deformable mirror are quantified by measuring the axial profile of the fluorescent beads (Fig. 4.6 b,c).

The axial separation of the image planes, denoted as ΔZ , is measured to be 739 ± 25 nm, matching the theoretical separation calculated as:

$$\Delta Z = \frac{L}{M^2} \frac{n_{sample}}{n_{bs}}, \quad (4.1)$$

Where M is the magnification before the image plane beam splitter, L is the interval between the beamsplitters, n_{bs} and n_{sample} are the refraction index of the sample and the beamsplitter. The focal shift caused by the deformable mirror can be expressed as:

$$\delta Z = -369 Z_5 nm \quad (4.2)$$

The focal shift with $Z_5 = 0.01$ is almost exactly half of the separation of the image planes. In the actual experiment, 4 focal shifts are obtained by decreasing z_5 from 0.01 to -0.005, resulting in a total of $4 \times 3 = 12$ images with a z-interval of 185 nm.

4.3.2 Multi-scan SIM imaging

SIM imaging of the fluorescent beads using the multi-scan imaging approach was conducted and the results are shown in (Fig. 4.7). The defocus mode of the deformable mirror was adjusted from -0.1 to 0.1 in intervals of 0.0025 to scan over a $6\mu m$ range. It's important to note that, unlike classical 3D-SIM approaches where the sample moves axially and the illumination pattern remains fixed in relation to the focal plane, in our scheme the illumination pattern is fixed in relation to the sample and volumetric imaging is achieved by moving the

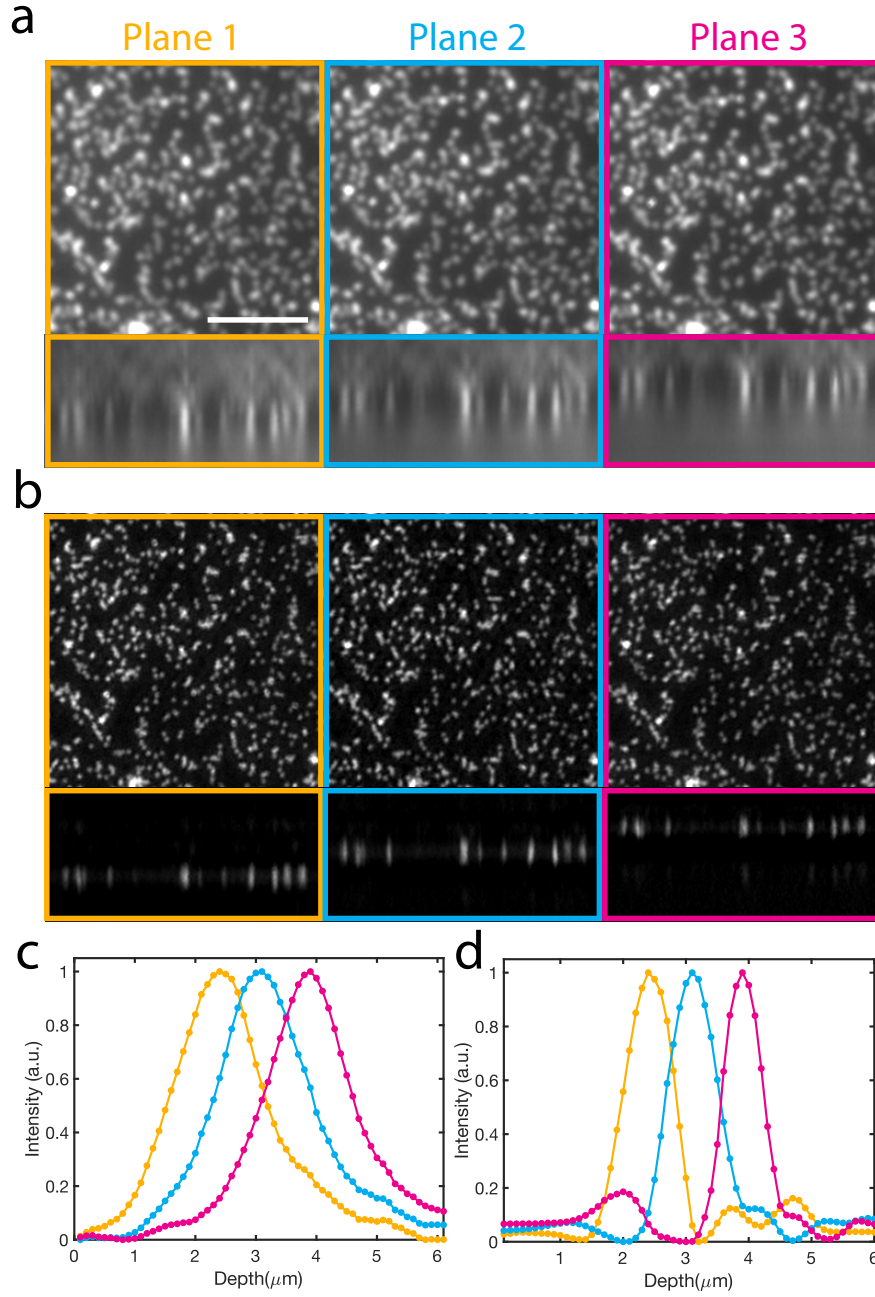


Figure 4.7: **Multi-scan 3D-SIM imaging of fluorescent beads.** (a) WF images of fluorescent bead sample. (b) SIM images of fluorescent bead sample. (c) Z-axis profiles of WF imaging at different image planes. (d) Z-axis profiles of SIM imaging at different image planes. Note the color coordination of the images of beads in (a,b) and the profiles in (c,d). Scale Bar: $5\mu\text{m}$.

focal plane axially. Consequently, direct application of multi-layer 3D-SIM reconstruction methods, such as open-3DSIM (Cao et al. (2023)), is not feasible for our reconstruction. Instead, we utilized FairSIM (Müller et al. (2016)) for single-slice SIM reconstruction, which still enables optical sectioning.

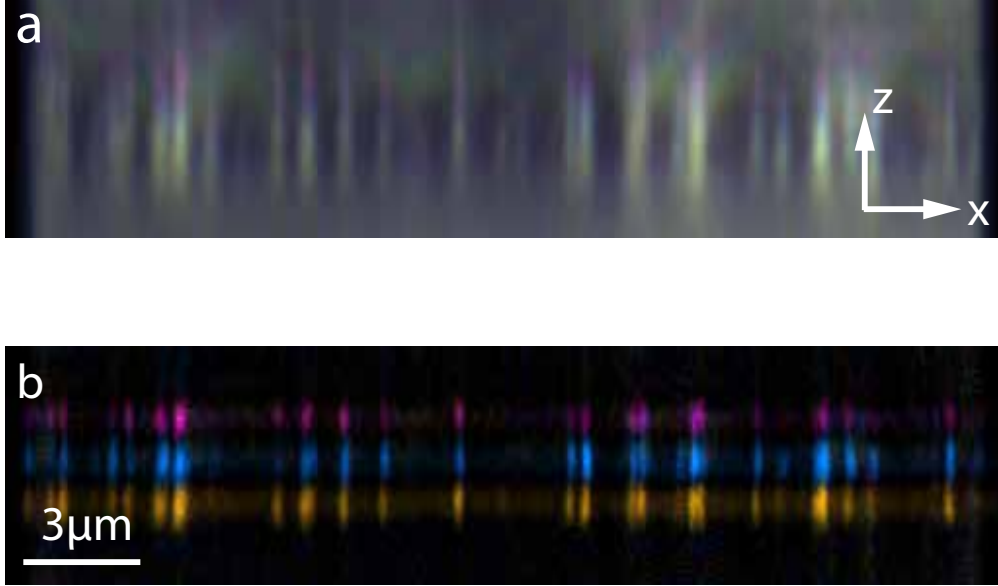


Figure 4.8: **Superimposed WF and SIM images from different image planes.** (a) superimposed lower pannel images of Figure 4.7a. (b) superimposed lower pannel images of Figure 4.7b.

As shown in Figure 4.7, compared to wide-field imaging, SIM imaging exhibits significant resolution improvement not only in the lateral direction but also in the axial direction. The full width at half maximum (FWHM) of the beads' profile along the z-axis is measured as $1.68 \pm 0.07 \mu m$ in wide-field imaging, while it is $0.91 \pm 0.11 \mu m$ in SIM imaging; approximately a $1.85 \times$ improvement in resolution along the z-axis. Additionally, the images of each individual image plane show great congruence, i.e., the aberrations between different image planes are negligible.

The improvement in z-axis resolution can be further observed in Figure 4.8, where the x-z images of the three imaging planes are displayed in the same image. Here, the beads' images are color-coded with the same colors used to plot the profiles in Figure 4.6 to indicate

the image plane. In wide-field imaging, the profiles of the beads in the three image planes are largely overlapped and cannot be distinguished. In contrast, SIM imaging clearly shows the three bead layers with clearly distinguished profiles.

4.4 MS-SIM imaging of cells

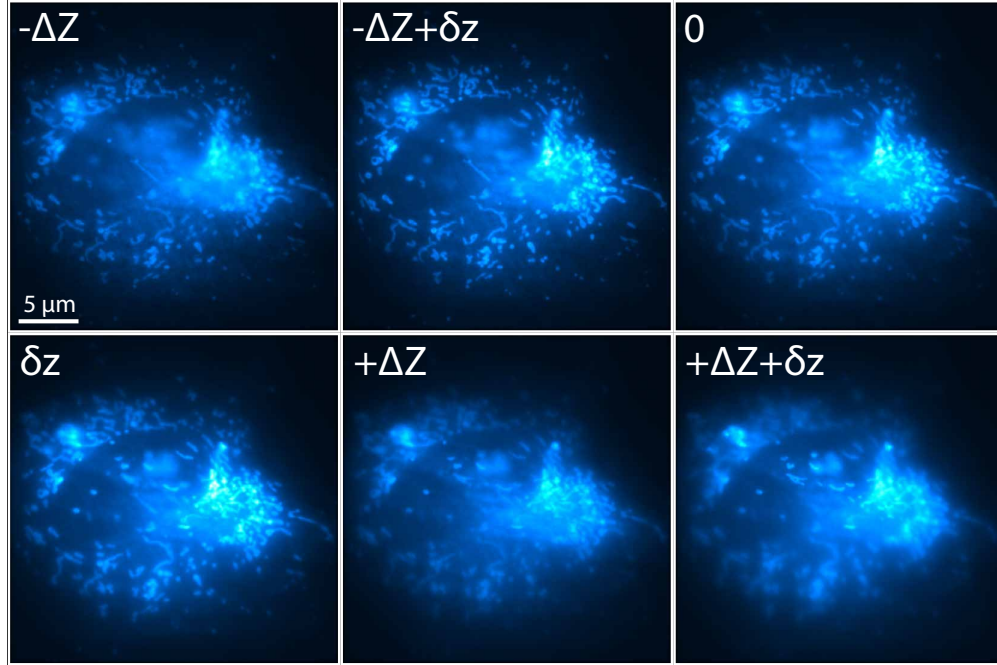


Figure 4.9: **Multi-scan wide field imaging of cell mitochondria.** $\Delta Z = 740$ nm, $\delta Z = 370$ nm.

After calibrating the focal plane locations (spacing) using fluorescent bead samples, MS-SIM volumetric imaging can be applied to fixed or live cell samples. Figures 4.9 and 4.10 show a six-slice stack of images of mitochondria in human BJ fibroblast cells. In wide-field imaging each image slice contains strong out-of-focus light. Although some information can be inferred from the blurriness of the image content, the wide-field image cannot provide accurate information about the distribution of mitochondria in the axiao direction. In contrast, SIM imaging shows very sharp optical sectioning and rejects most of the out-of-focus light. As a result, a clear distribution of mitochondria along the z-axis can be recorded with

great fidelity.

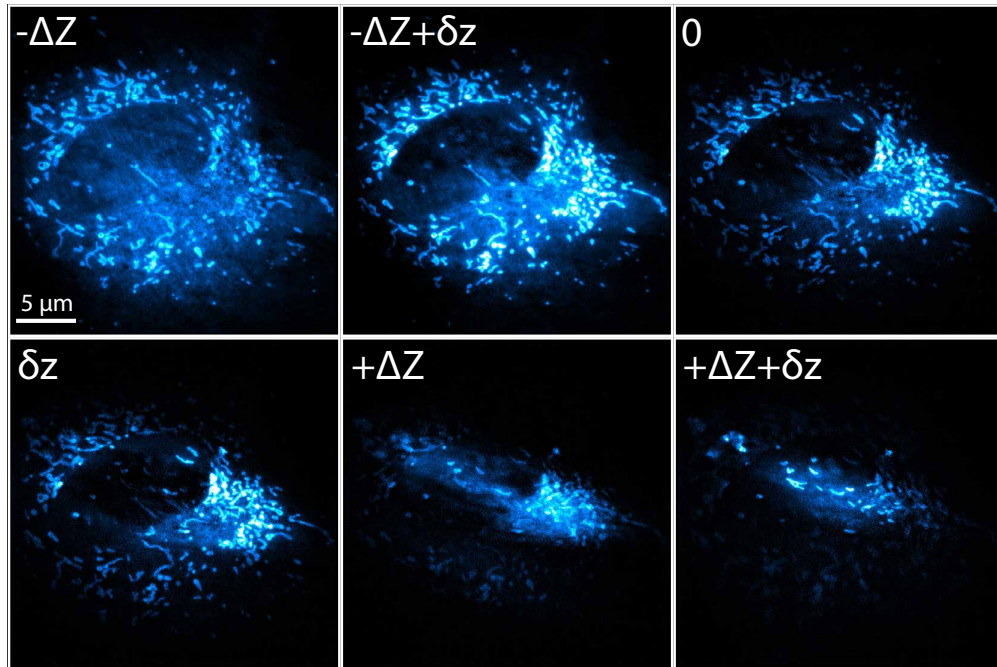


Figure 4.10: Multi-scan 3D-SIM imaging of cell mitochondria. $\Delta Z = 740\ \text{nm}$, $\delta Z = 370\ \text{nm}$.

4.5 Discussion and Conclusions

In this work, we introduced a novel approach to conduct high-speed volumetric SIM imaging by combining remote focusing for rapid z-axis scanning and an image plane beamsplitter for simultaneously imaging multiple focal planes. With this multi-scan scheme, we achieved high-speed imaging of three focal planes simultaneously and demonstrated this method on both single-layer bead samples and 3D live cell samples. Unlike previous approaches such as multi-focus SIM (Abrahamsson et al. (2017); Descloux et al. (2020); Senftleben et al. (2024); Johnson et al. (2022)), we did not aim to achieve a large number (e.g., $n \geq 9$) of simultaneously imaged focal planes. Instead, we opted for a moderate number of planes ($n = 3$) to strike a balance between speed and signal-to-noise ratio (SNR). Additionally, remote focusing was employed to scan between individual focal planes to achieve dense sampling of a 3D volume.

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CHAPTER 5

GDMD-SIM: EASILY SCALABLE MULTI-COLOR DMD-SIM

5.1 Introduction

The last 20 years have witnessed the explosive emergence of super-resolution microscopy (Schermelleh et al. (2019); Bond et al. (2022)). Among all the techniques, Structured Illumination Microscopy (SIM) provides a near-ideal balance between spatial and temporal resolution, low photodamage, optical sectioning capability, and no limitation on choice of dye (Schermelleh et al. (2019); Chen et al. (2023); Wang et al. (2023); Bond et al. (2022); Wang et al. (2022)). These advantages make SIM an ideal choice for super-resolution microscopy studies of live biological system. Yet, its adoption has been limited in great measure due to severe complications in its illumination setup.

SIM typically utilizes sinusoidal patterned illumination to shift unresolvable high spatial-frequency information into lower frequencies that can be captured within the microscope optical transfer function (OTF) (Gustafsson (2000)). Initially, diffraction gratings were used to generate the interference patterns for illumination (Gustafsson (2000); Gustafsson et al. (2008)). Then liquid crystal on silicon (LCoS) become widely adopted to generate structured illumination patterns due its higher speed and easier implementation (Young et al. (2016); Markwirth et al. (2019); Zhao et al. (2021); Luo et al. (2023)). In recent years, digital micromirror devices (DMD) were increasingly applied in SIM pattern generation (Sandmeyer et al. (2021); Li et al. (2020); Lachetta et al. (2021a); Li et al. (2017)). A DMD consists of an array of micro-mirrors that can be individually tilted along their diagonals into an "on" or "off" state, thus forming a binary reflectivity pattern. Compared with a LCoS, a DMD has the advantage of lower cost and orders of magnitude higher refresh rate thus becoming an attractive solution for high-speed SIM imaging. However, the application of DMDs in SIM is hindered because the tilted mirror array structure leads to a blazed grating diffraction effect

that causes strong angular dispersion for different excitation wavelengths (Chakrova et al. (2015)). To obtain an output with the desired angle and diffraction efficiency, one needs to precisely align the input angle individually for each excitation wavelength. This blazed grating effect makes the implementation of multiple excitation wavelengths in DMD-SIM exceedingly difficult.

Recently, DMD-SIM approaches with 2 or 3 coherent excitation beams have been demonstrated. However, they either require that the wavelengths have an integer ratio (e.g. 488 nm: 650 nm $\approx$ 3:4) (Lachetta et al. (2021b); Brown et al. (2021)) or add the complication of setting up individual light paths for different excitation wavelengths (Brown et al. (2021)), which is difficult to scale to more color channels. Alternatively, incoherent light sources that are free from the diffraction effect have been used to obviate this problem (Dan et al. (2013); Qian et al. (2015)). Unfortunately, this leads to decreased contrast of SIM patterns and hence inferior resolution (Li et al. (2020); Brown et al. (2021)). A solution without these complications and compromises is highly desirable.

In this letter, we present a novel multi-color DMD-SIM imaging design that overcomes the aforementioned limitations by introducing dispersion opposite to that of the DMD illumination system. A blazed grating is added to counteract the dispersion effect of the DMD. We show that this system can work with a wide range of laser illumination wavelengths commonly used for biological imaging without any specific (e.g. wavelength) requirements. We demonstrate the capability of this grating-DMD-SIM (gDMD-SIM) system by conducting three and four-color SIM super-resolution imaging of fluorescent beads and live cell samples, respectively. Benefiting from the “one-stop” nature of the dispersion compensation, our system can be easily scaled to even more wavelengths channels with minimal effort.

5.2 Dispersion compensation for a DMD

5.2.1 Diffraction characteristics of DMD

The periodic discrete element structure of the micro-mirror array makes the DMD behave as a diffraction grating. As Fig. 1A shows, the micro-mirror array can be considered as a 2-dimensional blazed grating, with a line spacing of $\delta = 7.56 \mu\text{m}$ in both directions. Each micro-mirror is tilted by an angle of $\gamma = 12^\circ$ along its diagonal. In practice, we rotated the whole DMD by 45° about its surface normal (i.e., z axis) so that the tilting is along the y axis (i.e., vertical axis).

Fig. 5.1 shows a detailed ray tracing schematic of the incident and diffracted rays of the DMD. In this coordinate system, the lattice vector $\vec{d}$ joining two adjacent micro-mirrors is:

$$\vec{d} = \left(\frac{\sqrt{2}}{2}\delta, \frac{\sqrt{2}}{2}\delta, 0 \right). \quad (5.1)$$

The incident ray $\vec{\alpha}$ and diffracted ray $\vec{\beta}$ are defined as

$$\vec{\alpha} = -(\sin \theta_0 \cos \phi_0, \sin \theta_0 \sin \phi_0, \cos \theta_0), \quad \vec{\beta} = (\sin \theta_1 \cos \phi_1, \sin \theta_1 \sin \phi_1, \cos \theta_1), \quad (5.2)$$

where (θ_0, ϕ_0) are the angles of $\vec{\alpha}$ between the z and x axis, and (θ_1, ϕ_1) are the angles of $\vec{\beta}$ between the z and x axes. The optical path difference between two adjacent micro-mirror pixels are given by:

$$\Delta = \vec{d} \cdot \vec{\alpha} + \vec{d} \cdot \vec{\beta} = \frac{\sqrt{2}}{2}\delta(\sin \theta_1(\cos \phi_1 + \sin \phi_1) - \sin \theta_0(\cos \phi_0 + \sin \phi_0)), \quad (5.3)$$

and the diffraction pattern is given by the grating equation:

$$\Delta = m\lambda_{ex}, m \in \mathbb{Z}, \quad (5.4)$$

where m is the diffraction order. At the blaze condition, one of the diffraction orders should overlap with the direct reflection of a single micro-mirror, that is:

$$\theta_0 - 2\gamma = \theta_1, \quad \phi_0 = -\phi_1, \quad (5.5)$$

and this will lead to maximum optical power concentrated to the single diffraction order.

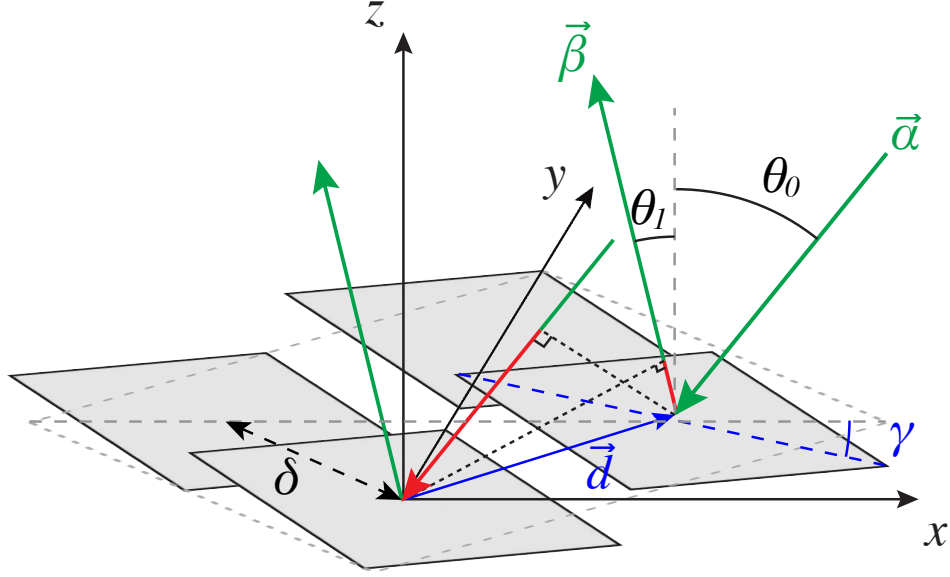


Figure 5.1: **Schematic of light propagating onto and from a DMD.** including angles of incidence and diffraction. See text for details of the symbols. The z axis is perpendicular to the plane of the DMD and x and y axes are in the plane of the DMD. This coordinate system is the same as in Fig 1A. The red line segments represent the optical path difference of incident rays ($\vec{d} \cdot \vec{\alpha}$) or diffracted rays ($\vec{d} \cdot \vec{\beta}$) for two adjacent micro-mirrors.

In practice, in order to maintain the the parallelism between the optical path and optical table, we constrain the incident light to be in a plane perpendicular to the y axis, i.e. $\phi_0 = 0$. In addition, to make the blaze diffraction parallel to the optical axis, a blaze condition at $\theta_1 = 0^\circ$ will be ideal. This leads to $\theta_0 = 2\gamma = 24^\circ$. Inserting these two constraints into the diffraction equation 5.4 gives the wavelengths of excitation light that satisfy the blazing condition:

$$\lambda_{ex} = -\frac{\sqrt{2}\delta \sin 2\gamma}{2m}, m \in \mathbb{Z}. \quad (5.6)$$

Apparently, the blaze condition can only be fulfilled with certain discrete wavelengths. In Table 5.1, we list some of those wavelengths that give rise to the blaze diffraction directed perpendicular to the DMD surface. Note that common laser wavelengths do not exactly match, which means the setup will then deviate from the ideal blazing case. In our system, we set the incident angle of the 532 nm beam on DMD to be $\theta_0 = \theta_{0G} = 23.46^\circ$ and use the $m = -4$ order diffraction at $\theta_1 = \theta_{1G} = 0^\circ$ as the illumination beam that goes into the microscope.

Blaze order	Wavelength (nm)
-2	1087.2
-3	724.8
-4	543.5
-5	434.9
-6	362.4

Table 5.1: Blaze orders and the corresponding blaze wavelengths that satisfy equation 5.6.

5.2.2 Dispersion compensation for a DMD

The lattice structure of the micro-mirror array makes a DMD a 2-dimensional blazed grating with a blaze angle ψ and line spacing δ in both directions. In our setup, the DMD is rotated 45° about its surface normal in order to make the rotating axis of the micro-mirrors perpendicular to the optical table. The diffraction beams that lie in the common horizontal plane with the input beam follows the grating equation:

$$\frac{\sqrt{2}}{2}\delta(\sin \theta_1 - \sin \theta_0) = m\lambda_{ex}, m \in \mathbb{N} \quad (5.7)$$

where θ_0 and θ_1 are the incident and diffracted angles, respectively, and m is the order of diffraction (Fig. 5.2A).

A key condition in our design is to have the plane of the DMD be parallel to the sample plane. At the blaze condition, one of the diffraction orders should overlap with the direct

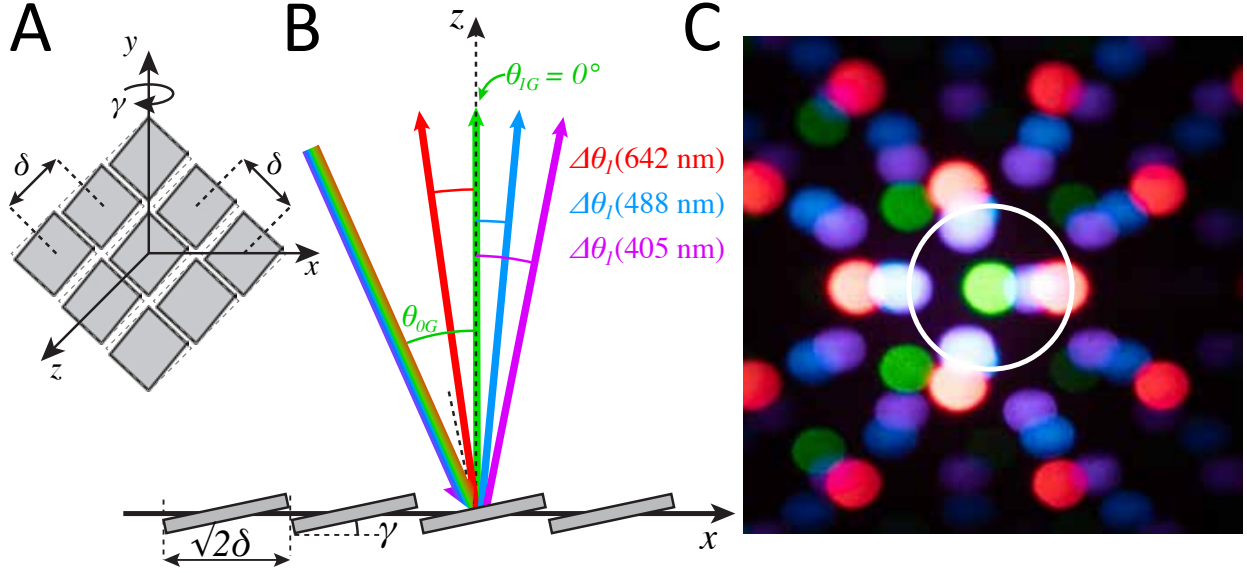


Figure 5.2: **Schematic and demonstration of blazed grating effect of DMD.** (A) Coordinate system and definitions. (B) Angular dispersion effect of DMD. (C) Photographed diffraction pattern of 4 colored beams (red, green, cyan, violet) without compensation by the grating. The white circle indicates the back aperture of the microscope.

reflection of a single micro-mirror, i.e., $\theta_0 - 2\gamma = \theta_1$, leading to maximum optical power concentrated to the single diffraction order. In practice, a blaze condition at $\theta_1 = 0^\circ$ would be ideal to make the blaze diffraction parallel to the optical axis. Adding these two constraints to equation 5.7, the wavelengths of excitation light that satisfy the blaze condition are:

$$\lambda_{ex} = -\frac{\sqrt{2}\delta \sin 2\gamma}{2m}, m \in \mathbb{N} \quad (5.8)$$

Table 5.1 shows some of the wavelengths that satisfy equation 5.8. In our case, the green laser with 532 nm wavelength is close to the blazing condition at $m = -4$ with $\theta_0 = \theta_{0G} = 23.46^\circ$ and $\theta_1 = \theta_{1G} = 0^\circ$. However, as seen in equation 5.7, the diffraction angle is highly dependent on the wavelength. At the same incident angle, the other lasers will be deviated from the optical axis by an angle of $\Delta\theta_1(\lambda_{ex})$, as seen in the color photograph of the diffraction patterns shown in Figure 5.2C.

To address this problem, an additional blazed grating is used to counteract the diffraction

effect of DMD. With an incident angle of ψ_0 , the first order diffraction of the grating follows the equation (Giles et al. (1973)):

$$d(\sin(\psi_{1G} + \Delta\psi_1) - \sin\psi_0) = \lambda_{ex} \quad (5.9)$$

where d is the line space of the grating, ψ_{1G} is the diffraction angle of the 532 nm green beam, and $\Delta\psi = \Delta\psi(\lambda_{ex})$ are the deviations of other wavelength beams relative to the green beam (Fig. 5.3A). These deviations propagate through the relay lens pair and lead to the same deviations of the incident angles onto the DMD. The resulting grating equation of the DMD becomes:

$$\frac{\sqrt{2}}{2}\delta(\sin(\theta_{1G} + \Delta\theta_1) - \sin(\theta_{0G} + \Delta\psi_1)) = m\lambda_{ex} \quad (5.10)$$

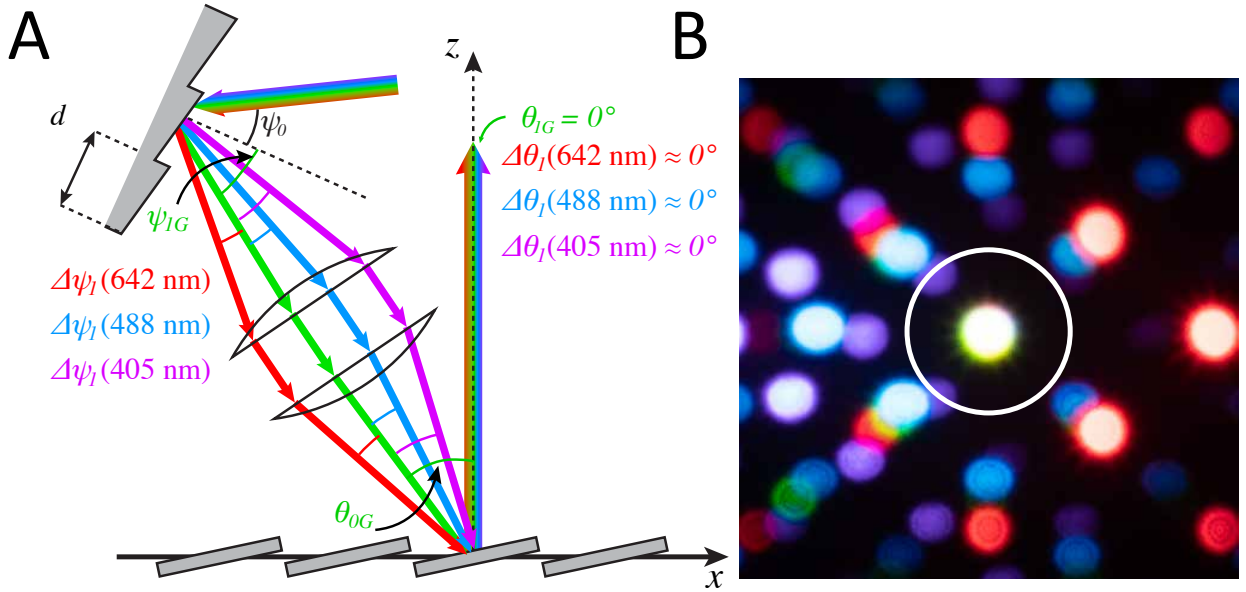


Figure 5.3: **Schematic and demonstration of grating dispersion compensation of the DMD blazed grating effect.** (A) Angular dispersion effect of DMD with compensation by the grating. (B) Photographed diffraction pattern of 4 colored beams with compensation by the grating. The white circle indicates the back aperture of the microscope.

Combining equations 5.9 and 5.10, we can determine the values of $\Delta\theta_1(\lambda_{ex})$ as a function of ψ_0 (Fig. S2). Our setup uses a 600 lines/mm grating. When $\psi_0 = 21.41^\circ$, then $\Delta\theta_1$ for 405

nm, 488 nm and 642 nm beams are 0.087° , 0.001° , 0.160° respectively, which are within the tolerance for alignment. As shown in the photograph of Figure 5.3B, after adding the grating for dispersion compensation, the -4th order diffraction of all 4 lasers are well overlapped in the center white spot and propagate colinearly along the optical axis.

5.3 Experimental methods

5.3.1 Choice of the blazed grating

To determine the ideal parameter (i.e., period, d , and blaze angle, ν) of the blazed grating, we can first calculate the dispersion of a grating as $\frac{m_1}{d \cos(\psi_1)}$, where ψ_1 is the diffraction angle of the grating, m_1 is the diffraction order, and d is the grating period. The first order is desired to achieve the maximum efficiency. For a DMD with pixel size δ , the dispersion along its diagonal direction can be similarly expressed as $\frac{2m}{\sqrt{2}\delta \cos(\theta_1)}$, where θ_1 and m are the diffraction angle and order of the DMD, respectively. In our case, θ_1 is equal to 0° and m is equal to -4 as mentioned in supplementary Section 1. In a small wavelength range, the dispersion of the blazed grating and the DMD need to have opposite values to achieve dispersion compensation:

$$\frac{1}{d \cos(\psi_1)} + \frac{2m}{\sqrt{2}\delta} = 0. \quad (5.11)$$

This equation only works in a small range of wavelengths and cannot be used to exactly determine the grating parameter for our multi-color imaging application. However, it indicates that a grating with relatively small line density (i.e. large d), such as 300 or 600 lines/mm, has to be chosen to not over compensate the dispersion caused by the DMD. In our work we chose the line density to be 600 lines/mm since the choices of commercially available 300 lines/mm gratings are limited.

After the d is determined, the incident angle ψ_0 to achieve precise angle corrections for

all 4 wavelengths used in our study can then be determined by numerical calculation from equations 3 and 4. As shown in Figure 5.4, $\psi_0 = 21.41^\circ$ when the line density is chosen to be 600 lines/mm. The diffraction angle ψ_1 can then be calculated to be $\psi_1 = 43.18^\circ$ from equation 3. To satisfy the blazed condition an ideal blaze angle ν for the blazed grating can be calculated as

$$\nu = \frac{\psi_0 - \psi_1}{2} = -10.88^\circ. \quad (5.12)$$

The negative sign is an indication of the orientation of the blazed grating (as illustrated in Figure 2). In our work, we chose a blazed grating with 600 lines/mm grooves and a blaze angle of 8.62° (Thorlabs GR13-0605) to most closely match the ideal parameters. Although it does not perfectly satisfy the blaze condition resulting in to energy loss, this mismatch does not affect the dispersion compensation function for our application. Since SIM has a modest requirement for illumination intensity, our setup can still achieve high quality live cell SIM imaging.

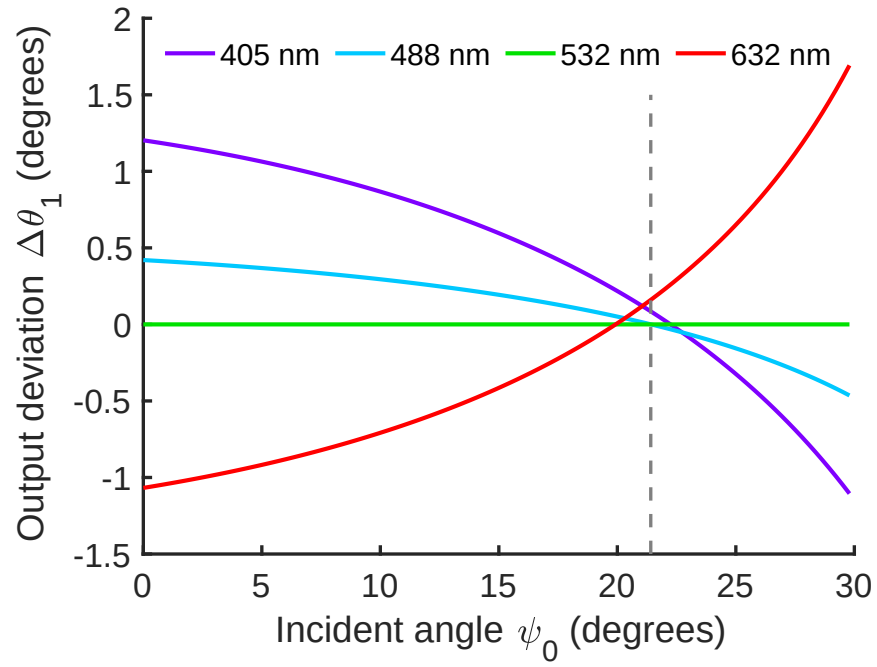


Figure 5.4: Calculated output deviation $\Delta\theta_1$ of different wavelength beams versus the incident angle ψ_0 onto the blazed grating. The calculation is based on the equations 3 and 4.

5.3.2 Microscopy setup

A schematic of the gDMD-SIM optical setup is shown in Fig. 5.5A. Four lasers with wavelengths 405, 488, 642 nm and 532 are used as excitation light sources. The lasers are merged, expanded, and then dispersed into monochrome beams by a blazed grating (600 lines/mm, 500 nm blaze). The first-order diffraction of each wavelength beam goes through a pair of relay lenses with unitary magnification and then converges at the plane of the DMD (pixel pitch $\delta = 7.56 \mu\text{m}$, tilt angle $\gamma = 12^\circ$). The patterns generated by the DMD are projected to the sample plane after propagating through a pair of relay lenses, a tube lens and the objective lens. A multi-band dichroic mirror and a bandpass filter set were used to separate the excitation and emission light. A sCMOS camera was used to record the fluorescence images. More detailed information about the hardware and setup are provided in Table S2.

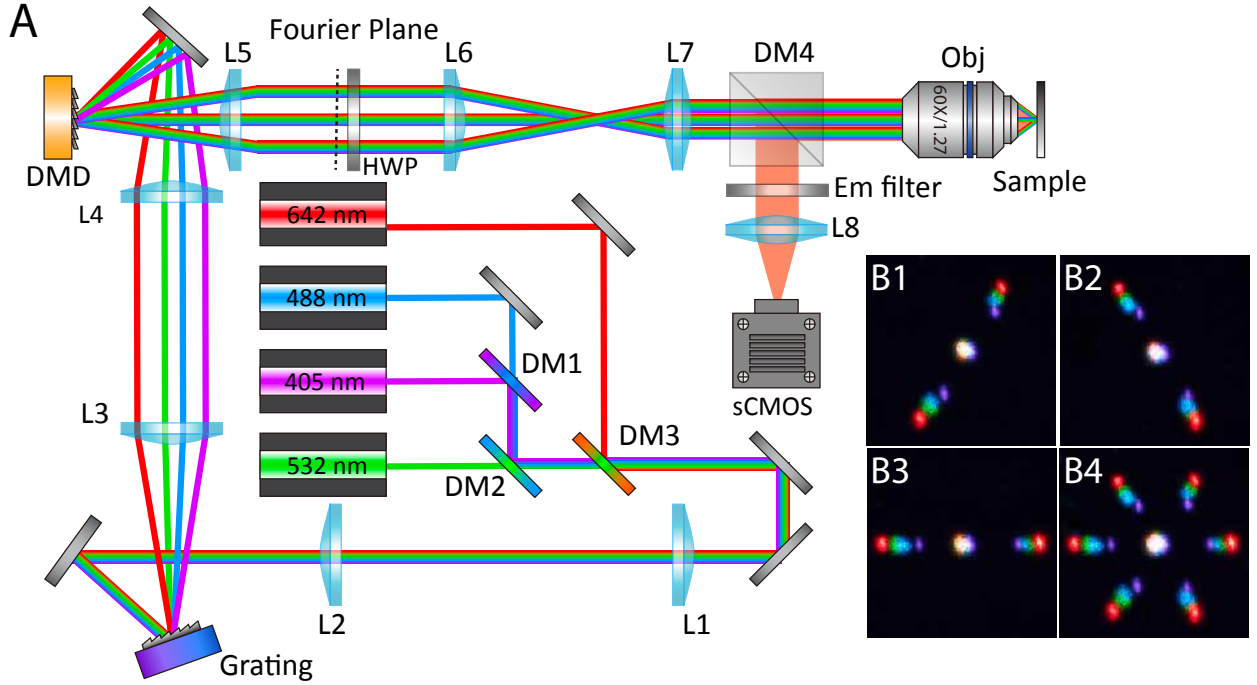


Figure 5.5: **Experiment setup of the multicolor gDMD-SIM.** (A) Schematic of the optics setup. (B1-B3) Photographed diffraction patterns at the Fourier plane when 3 different angles of SIM patterns are displayed by the DMD. (B4) Merge of B1-B3.

When doing SIM imaging, striped binary patterns with (3) different angles and (6) phases

are displayed by the DMD in a time series. These binary patterns lead to 2 more diffraction orders around the collimated beams ($m = -4$ diffraction order) (Fig. 5.5B). The radial shifts of the ± 1 orders between different wavelengths, caused by the grating associated with the binary patterns, are innocuous since they are symmetric about the optical axis. These three diffraction orders are collected by the microscope aperture and serve as the center (0) order and ± 1 orders forming the interference patterns at the sample plane. The same set of SIM patterns with the second-order spatial frequency of $2.86\mu m^{-1}$ (i.e. 350 nm) at the sample plane are used for all color channels, leading to a theoretical resolution improvement of $1.93\times$ (642 nm channel), $1.80\times$ (532 nm channel), $1.71\times$ (488 nm channel) and $1.60\times$ (405 nm channel). FairSIM, an ImageJ-based open source software Müller et al. (2016) was used to perform super-resolution SIM reconstruction. More detailed information about the SIM reconstruction are provided in Table 5.3.

5.4 Experimental results

5.4.1 *gDMD-SIM imaging of fluorescent beads*

To test the capability of the microscope, we first conducted 3-color SIM imaging of 100 nm diameter multi-color fluorescent bead samples by both wide-field and SIM imaging.

Compared with wide-field images (Fig. 5.6A), it can be seen that the SIM reconstructed images (5.6B) show significant spatial resolution improvement in all three color channels. Fourier ring correlation (FRC) analysis was conducted to quantitatively determine the resolution improvements. The image resolution is determined as when a FRC curve drops below a fixed threshold of $1/7$. SIM imaging and FRC analysis were conducted in 8 different fields of view. The spatial resolutions of wide-field/SIM images are $326 \pm 5/167 \pm 1$ nm (642 nm channel), $279 \pm 3/153 \pm 2$ nm (532 nm channel) and $242 \pm 4/142 \pm 2$ nm (488 nm channel), corresponding to resolution improvements of $1.93 \pm 0.03\times$, $1.83 \pm 0.03\times$ and $1.71 \pm 0.03\times$

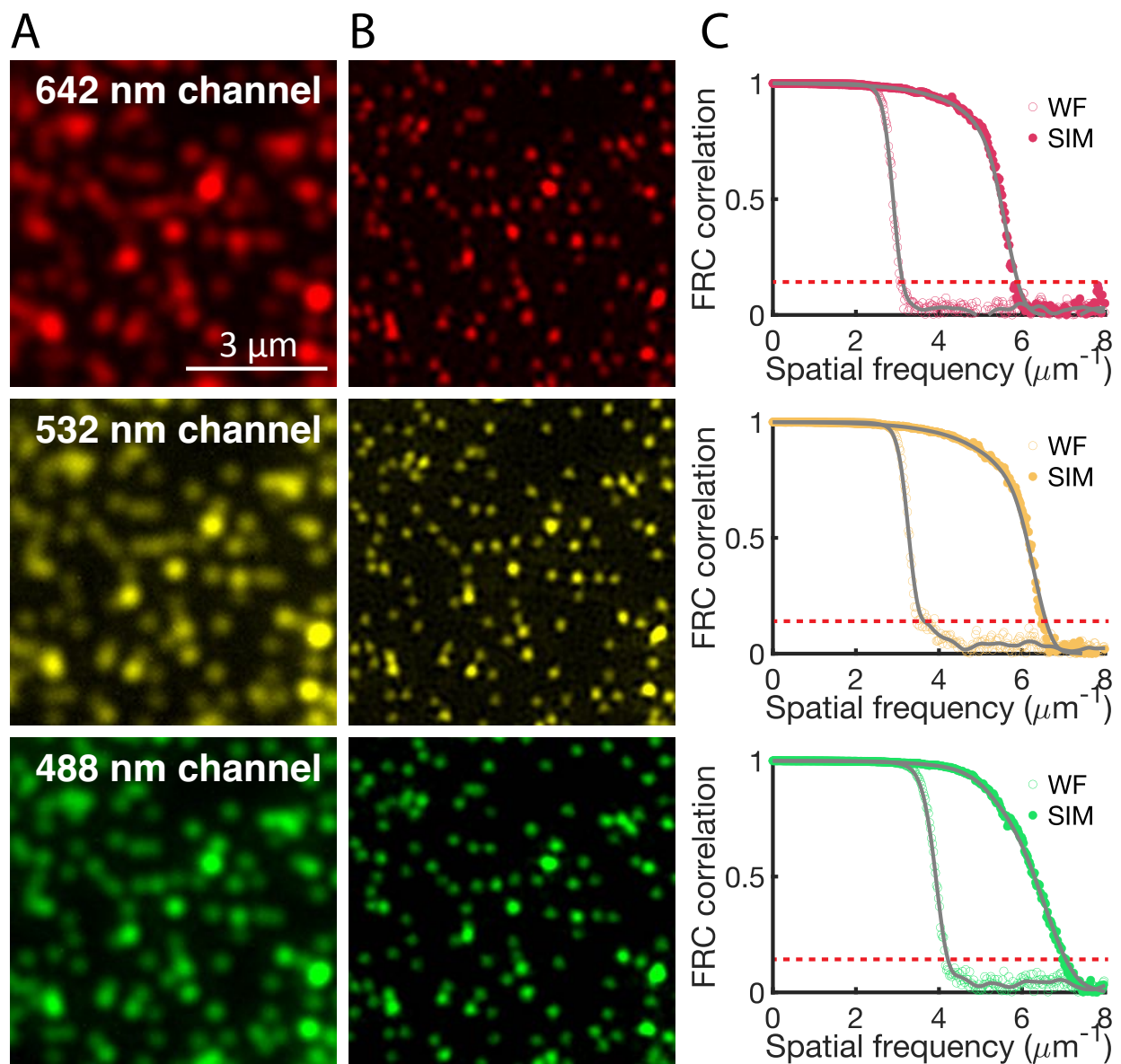


Figure 5.6: **3-color SIM imaging of fluorescent bead samples.** (A) Wide-field images of bead samples in 3 color channels. (B) SIM images of bead samples in 3 color channels (C) FRC correlation curves of wide-field and SIM images in 3 color channels.

respectively (Fig. 5.6C). These results match the theoretical calculation of the expected resolution improvement very well.

5.4.2 *gDMD-SIM imaging of cells*

We also performed 4-color SIM imaging of *live* human BJ fibroblast cells. Excitation wavelengths of 642 nm, 532 nm, 488 nm, and 405 nm were used to image tubulin, actin, mitochondria, and the nucleus labeled with Tubulin Tracker deep red, CellMask Orange, MitoTracker, and Hoechst, respectively. Figures 5.7A-E show the resulting fluorescence images with composite color channels. Some representative intensity profiles of individual structures in WF and SIM images are shown in Figures 5.7F-I. The SIM images show significantly superior quality compared with the WF images (Fig. 5.7A). The resolution of each color channel quantified by image decorrelation analysis Descloux et al. (2019) are 176 ± 7 nm (642 nm channel); 154 ± 3 nm (532 nm channel); 146 ± 4 nm (488 nm channel); 134 ± 6 nm (405 nm channel) (Fig. 5.8). These results match well with the theoretical calculation and the FRC analysis. This improvement allows the SIM images to reveal structural details that are indistinguishable in the WF images, such as adjacent microtubules (Fig. 5.7B & F), actin filaments (Fig. 5.7C & G), and mitochondria (Fig. 5.7D & H). The optical-sectioning of SIM also helps filter out the out-of-focus fluorescence signal and leads to a much clearer background compared to WF images (Fig. 5.7E & I). We further performed time series 4-color live cells SIM imaging (Fig. 5.9). The dynamics of different cellular structures, such as morphology change of mitochondria and fluctuation of microtubules, are well resolved.

5.5 Discussion and conclusions

In summary, we demonstrated a novel gDMD-SIM system that overcomes the blazed grating effect intrinsic to DMDs and enables multi-color imaging within a wide range of excitation wavelengths. A second blazed grating was placed relaying the different color excitation beams

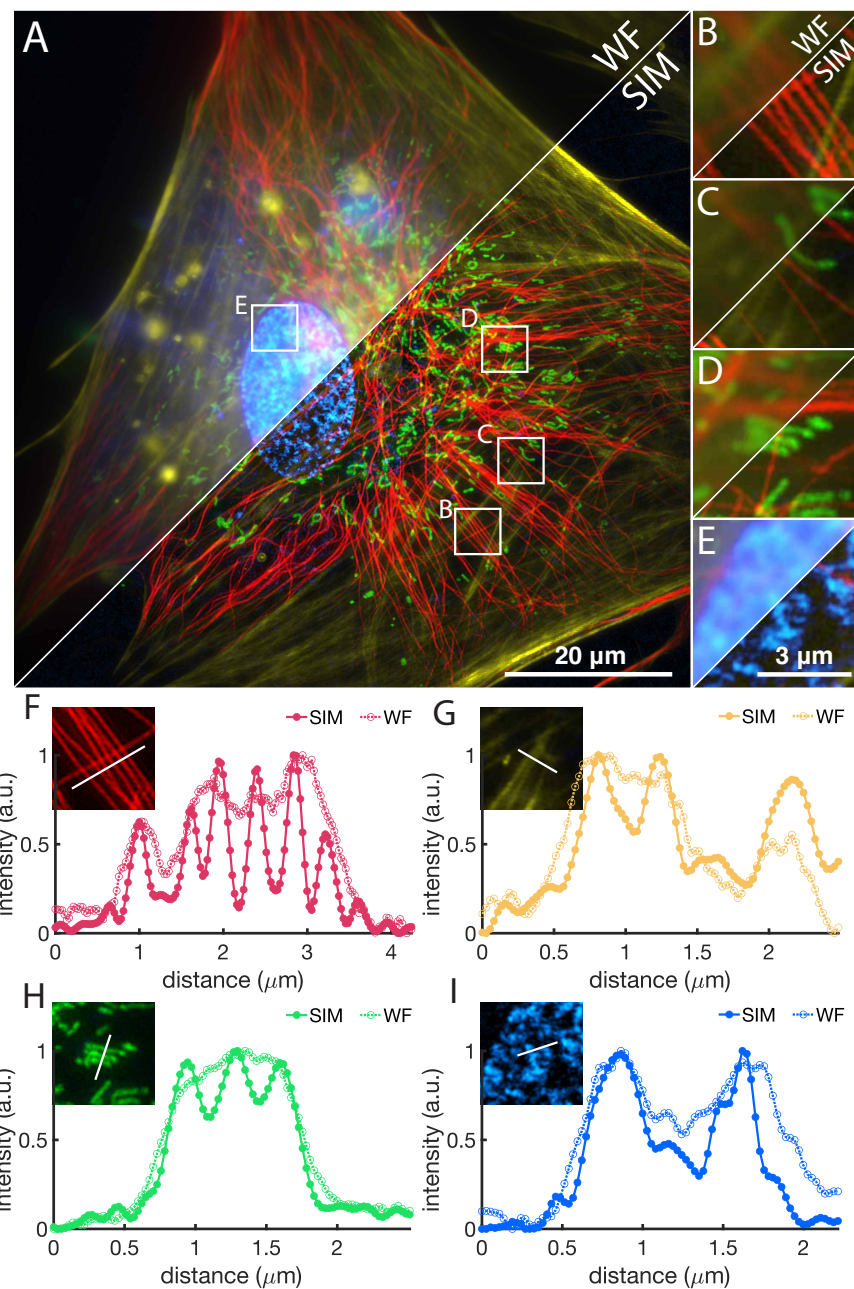


Figure 5.7: **4-color SIM imaging of live human BJ fibroblast cells.** (A) WF and SIM images of the microtubules (642 nm channel, red), actin (532 nm channel, yellow), mitochondria (488 nm channel, green), and nucleus (405 nm channel, blue). (B-E) WF and SIM images of the region of interest illustrated in A. (F-I) Comparison of normalized intensity profiles of WF and SIM images along the white line shown in the insets. (F) Intensity profiles of the microtubule channel in (B). (G) Intensity profiles of the actin channel in (C). (H) Intensity profiles of the mitochondria channel in (D). (I) Intensity profiles of the nucleus channel in (E). Exposure time for each raw image is 100 ms.

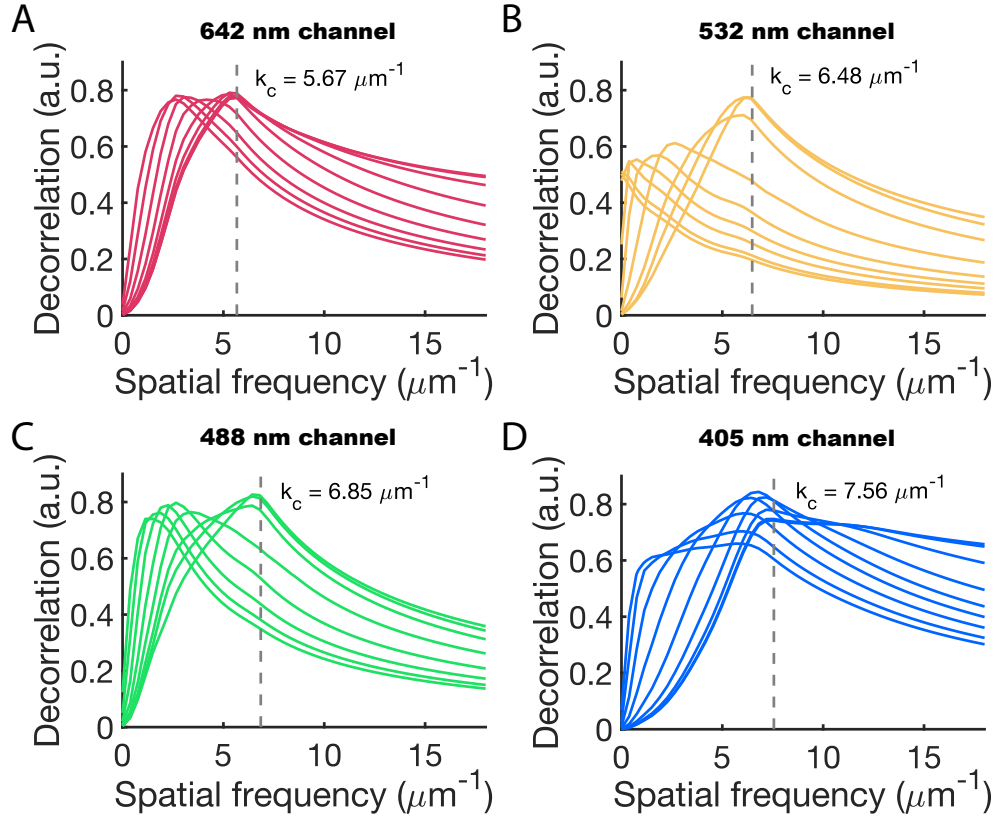


Figure 5.8: **Image decorrelation analysis of the four channels of live cell imaging.** 10 different cell images were analyzed. The resolution of each channel is determined to be: 176 ± 7 nm (642 nm channel); 154 ± 3 nm (532 nm channel); 146 ± 4 nm (488 nm channel); 134 ± 6 nm (405 nm channel). These results match well with the theoretical calculation and the FRC analysis presented and discussed in the main text.

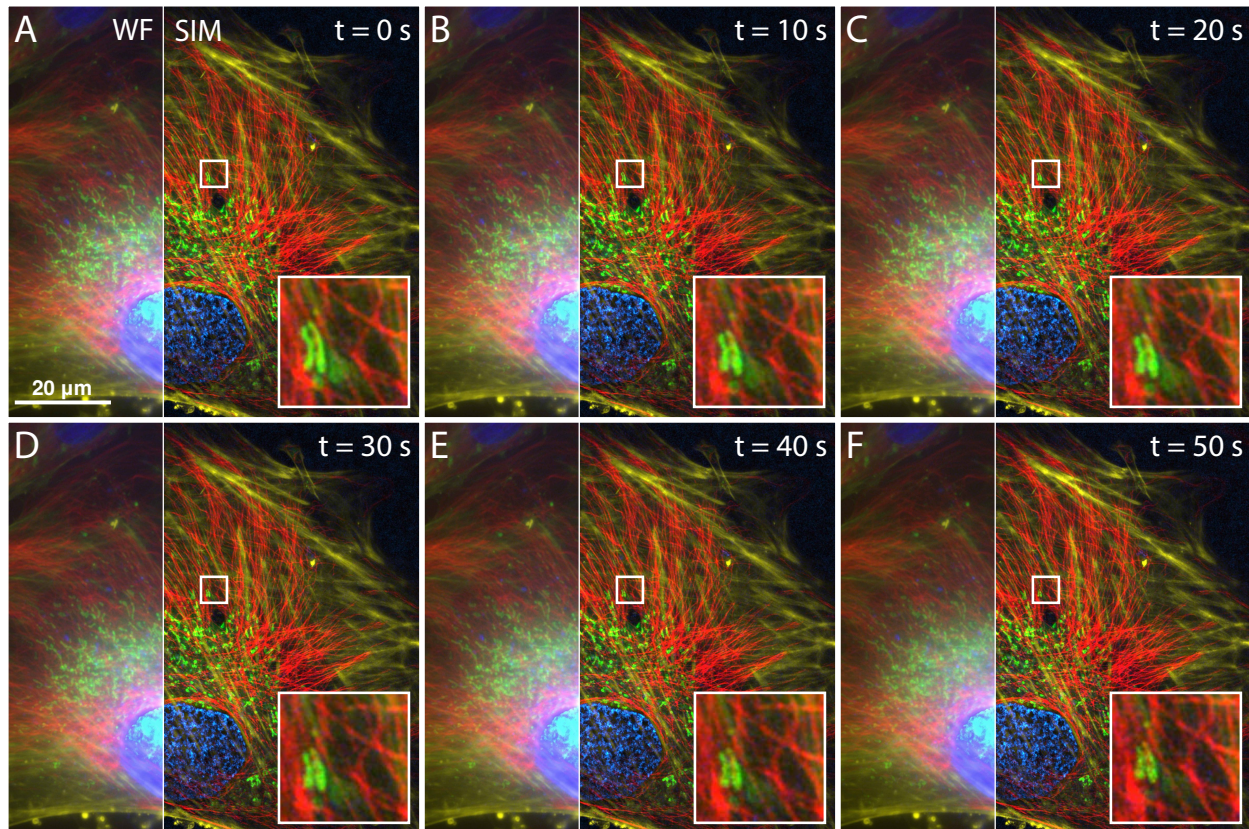


Figure 5.9: **Time series of 4-color SIM imaging of live human BJ fibroblast cells.** Exposure time for each SIM raw image: 30 ms. The insets show the fluctuation of microtubule fibers and the morphology changes of mitochondria during the time window. For the four-color live cell imaging shown, the $4 \times 18 = 72$ subimages required for SIM reconstruction, at present, limits the SIM frame rate to 0.4 Hz. The 6 images are shown in 10 second intervals and with similar intensity and SNR indicating good capability for longer time imaging.

to the DMD’s position. The grating introduces angular dispersion opposite to the DMD and thus canceling the output angle differences between different color excitation beams. Multi-color SIM imaging of fluorescent beads and subcellular structures was demonstrated. The gDMD-SIM method does not involve any mechanical movement or fine tuning of individual excitation light paths. Once the position and orientation of the grating is set, it immediately allows extension to more excitation channels without any extra effort other than collinearization of the beams.

gDMD-SIM could be further extended to volumetric imaging by using a spatial light modulator (SLM) functioning as a blazed grating for dispersion compensation. The SLM can accommodate additional modulation patterns, including patterns optimized for the correction of aberrations Gong and Scherer (2023). Such capabilities can facilitate imaging through thick 3D samples such as biological tissue.

We acknowledge that this gDMD-SIM method has relatively low excitation energy efficiency (Supplemental 1). Since the beams are not at the most ideal blazed condition of the grating and the DMD, the output energy of the excitation beams to be attenuated. Nonetheless, we note that SIM is well known for its modest illumination intensity requirement Wu and Shroff (2018); Zhao et al. (2021). In our system, the illumination intensities of the 4 excitation beams at the sample plane are: 12 W/cm² (642 nm), 20 W/cm² (532 nm), 16 W/cm² (488 nm) and 4 W/cm² (405 nm), which are in the typical range for SIM imaging Wu and Shroff (2018). As shown in the time series of live cell imaging (Figure S5), our system can maintain good image quality with a short exposure time of 30 ms. In our experiments, the image quality is not limited by the power of the light source, but instead the photo budget associated with photobleaching and phototoxicity.

Overall, the gDMD-SIM system presented here offers a simple and promising approach for multi-color DMD-SIM imaging, which is otherwise very challenging to achieve. This method features simple implementation and easy scalability to a wide range of color channels and

possess substantial potential for general and practicable adoption across diverse domains of biological research.

Appendix 5.A Energy efficiency

In the gDMD-SIM method, a suitable choice of the blazed grating and the input angle can successfully counteract the angular dispersion effect of the DMD and allow aligning all the 4 color beams to the center of the optical path after the DMD. However, this alignment does not fully meet the blaze condition of the blazed grating and the DMD, which leads to relatively low energy efficiency. As a reference, we measured the beam power we used for live cell imaging at several critical locations along the beam path, as shown in Table 5.2.

	L1 - L2	L3 - L4	L5 - L6	sample plane
642 nm	72 mW	25 mW	1.8 mW	1.2 mW
532 nm	20 mW	11 mW	3.0 mW	2.0 mW
488 nm	76 mW	48 mW	2.5 mW	1.6 mW
402 nm	62 mW	24 mW	0.6 mW	0.4 mW

Table 5.2: Illumination power of the laser beams at different locations. L1 - L2/L3 - L4/L5 - L6/ means the power was measured at the location between the lenses L1/L3/L5 and L2/L4/L6.

The 532 nm beam has the highest energy efficiency since it is closely aligned to the blazed condition of the DMD. The other 3 beams show significant energy loss, especially after the DMD, due to the deviation from their blaze condition. Despite this energy loss, in a $200\mu\text{m}$ diameter field of view, the illumination intensity still has a value of at least 4 W/cm^2 , which falls well into the common range for SIM excitation intensity Wu and Shroff (2018).

The deviation from blaze condition could also lead to uneven intensities of the ± 1 order beams for the SIM patterns, especially along the direction perpendicular to the y axis of the DMD. However, as we show in Figure 5.10, the max difference between the ± 1 order beams is $\sim 24\%$. Although this can lead to a decrease of the modulation amplitude of the SIM pattern, in our experiment we do not observe noticeable problem from this intensity

asymmetry.

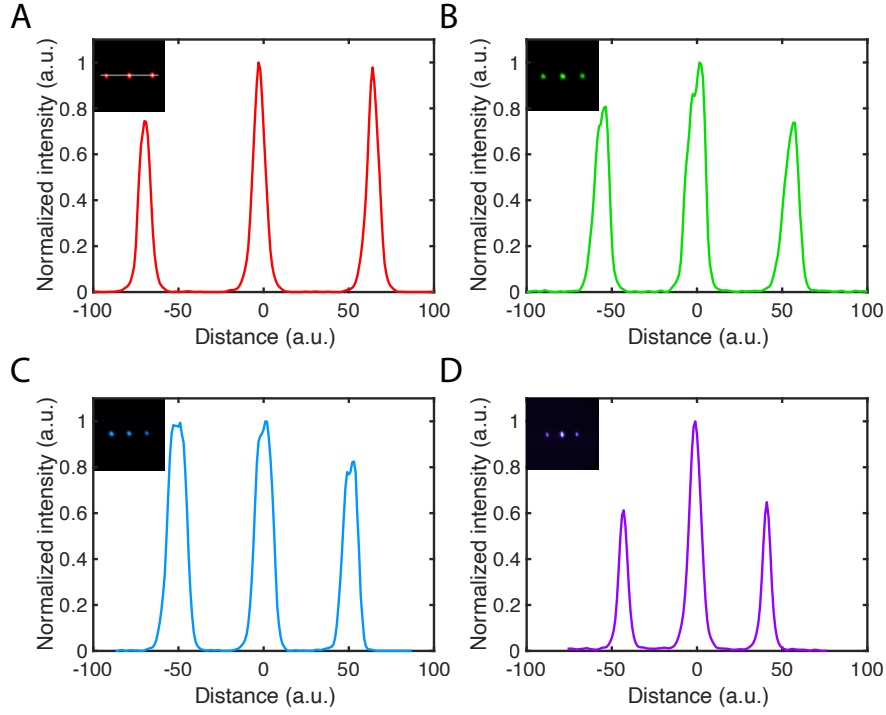


Figure 5.10: Normalized intensities of the ± 1 and 0 order beams for the 4 different wavelengths used in our study. The SIM pattern angle is chosen to be nearly paralleled to the y axis, in which case the diffracted beams show the strongest asymmetry. The intensities are measured along the gray line shown in the inset of A. The intensity ratio of the -1, 0 and +1 order beams are: (A) 642 nm channel: 0.74, 1, 0.98; (B) 532 nm channel: 0.8, 1, 0.74; (C) 488nm channel: 1, 1, 0.82; (D) 405nm channel: 0.61, 1, 0.65.

Appendix 5.B Hardware information

Component	Part number	Description
642 nm laser	Spectra-Physics, Excelsior One 642	100 mW maximum power
532 nm laser	Spectra-Physics, Millennia V	5 W maximum power
488 nm laser	Spectra-Physics, Excelsior One 488	100 mW maximum power
405 nm laser	Spectra-Physics, Excelsior One 405	100 mW maximum power
DM1	Semrock, Di03-R405-t1-25x36	414 nm long pass
DM2	Semrock, Di03-R488-t1-25x36	500 nm long pass
DM3	Thorlabs, DMSP550R	550 nm short pass
L1	Thorlabs, ACT508-100-A-ML	$f = 100$ mm
L2	Thorlabs, ACT508-400-A-ML	$f = 400$ mm
Grating	Thorlabs, GR13-0605	600/mm, 500 nm blaze
L3	Thorlabs, ACT508-200-A-ML	$f = 200$ mm
L4	Thorlabs, ACT508-200-A-ML	$f = 200$ mm
DMD	Texas instrument, DLP9000X VIS WQXGA	1600×2560 pixels
L5	Thorlabs, ACT508-180-A-ML	$f = 180$ mm
HWP	Thorlabs, AHWP10M-600	achromatic half wave plate
HWP mount	Newport, AG-PR100	rotation stage for the HWP
L6	Thorlabs, ACT508-250-A-ML	$f = 250$ mm
L7	Thorlabs, ACT508-300-A-ML	$f = 300$ mm
L8	Nikon, Eclipse-Ti (internal part)	$f = 200$ mm
Obj	Nikon, SR Plan Apo, 60X, 1.27 WI	
DM4	Semrock, Di01-R405/488/532/635-25x36	multi-edge dichroic mirror
Emission filter 1	Semrock, FF01-446/510/581/703-25	multi-band bandpass filter
Emission filter 2	Chroma, ET590/33m	590/33 nm bandpass filter
Emission filter 3	Semrock, FF01-525/39	525/39 nm bandpass filter
sCMOS	Photometrics, Kinetix	

Table 5.3: Information of the hardware components of the microscope setup. Here the HWP mounted on the motorized rotation stage was used to adjust the polarization of the beams in order to maximize the contrast of SIM patterns. Emission filter 1 is used for all channel imaging, while the emission filter 2/3 is added when imaging 532/488 nm channel to prevent signal cross talk.

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CHAPTER 6

μ SPLIT-SIM: VIRTUAL MULTI-CHANNEL SIM IMAGING

6.1 Introduction

Compared to label-free imaging, a major advantage of labeled imaging, such as fluorescence microscopy, is its ability to selectively identify objects of particular interest to the observer while excluding unrelated objects (Astratov et al. (2023)). For instance, if you want to image microtubules, it is much more preferable for your image to contain only microtubules without other cellular structures such as actin or mitochondria that could interfere with your observation of the microtubule structure. This selective imaging feature is so important that it has made fluorescence microscopy the most widely adopted technique for bioimaging research. In contrast, the application of label-free imaging techniques, such as phase-contrast or dark-field imaging, is largely limited to simple purposes, such as detecting cell morphology (Astratov et al. (2023)).

However, as biology research advances, what used to be the advantage of fluorescence microscopy begins to limit itself: since everything needs to be labeled before imaging, the total number of objects that can be imaged is confined within the limited bandwidth of available labels. Most fluorescent labels have an emission spectrum of approximately 100 nm within the range of 400 nm to 800 nm. For instance, the green fluorescent protein (GFP) has an emission spectrum ranging from 490 nm to 560 nm, while the red fluorescent protein (RFP) has an emission spectrum ranging from 550 nm to 670 nm. Another limitation comes from the light source. Different labels have different excitation spectra and likely require different light sources. For instance, GFP is typically excited by a 488 nm light source, while RFP is typically excited by a 562 nm light source. Although it is not a significant challenge to set up two laser sources for these two fluorescent proteins, it becomes much more challenging to find ten different light sources to image ten labels with different excitation

spectra. Clearly, the number of fluorescent labels cannot be increased indefinitely, as their excitation and/or emission spectra would overlap, causing the image content of different labels to mix together.

Nowadays, due to the limited choice of labels and lasers, most multi-color fluorescent microscopy have their color channels limited to 3 or 4, such as the gDMD-SIM (Gong et al. (2024)). In contrast, there could be hundreds or thousands of molecules or structures involved in a biological process, and it would be a significant bias to only selectively image a small portion of them. For this reason, the channel capacity of fluorescence imaging needs to be increased significantly.

Though it may sound like a contradiction given the aforementioned discussion, one of the most promising strategies to increase the channel capacity is to simultaneously image multiple labels in one channel. If n labels can be imaged simultaneously in one color channel, the total number of labels that can be imaged is then improved $n\times$. However, in this scenario, the problem reverts to the similar issue for label-free imaging: if multiple things are imaged in one channel, they will mix and interfere with each other for unambiguous identification. Fortunately, unlike label-free imaging where thousands of molecules and intercellular structures mix together, only n molecule or structure types are mixed together, and the value of n can be well-controlled to a reasonable value, e.g., 3 or 4. Computational, particularly machine learning, methods can then be devised to decompose this mixed image and separate each individual label.

Currently, there are two routes to conduct this image decomposition task: spectrum-based demixing and morphology-based demixing. For spectrum-based demixing, the mixed image is treated as a linear combination of a multi-channel image with different emission spectra as the coefficients. The mixed image can then be demixed pixel-by-pixel based on prior knowledge of the spectrum of the label (McRae et al. (2019); Cang et al. (2023); Jiang et al. (2023)). For morphology-based demixing, the image is not demixed pixel-by-pixel;

instead, it is decomposed based on the morphology of the image content (Guo et al. (2020); Cross-Zamirski et al. (2022); Christiansen et al. (2018); Ounkomol et al. (2018); Somani et al. (2022); Jin (2023)). This typically requires a deep learning model to learn the features of the structures that need to be recognized. This method is not limited to image decomposition of mixed channel images but can also be applied to label-free images to selectively recognize structures of interest; therefore, it is also referred to as "virtual staining." Compared to spectrum demixing, virtual staining relies on the structural features captured in the images for specific identification. Therefore, it is only suitable for objects with clearly defined structures or morphologies, such as mitochondria, tubulin (microtubule), cell membranes, etc., and not applicable to small molecules, which typically have point-like images. Despite these limitations, the fact that virtual staining is based on shape rather than spectrum makes it much more robust and transferable to different imaging setups, offering huge potential for wider application.

In this Chapter, We demonstrate a combination of μ Split (Ashesh et al. (2023)), a morphology-based image decomposition deep learning model, with multi-color SIM imaging to achieve simultaneous 3-color imaging. A large SIM image dataset of mitochondria, microtubules, and actin is acquired and used to train the μ Split model to learn the structural features of each subcellular structure. The model then demonstrates very good performance in image decomposition of mixed (gray scale not color) images of the three different structures. This method achieves multi-color imaging with only one channel and has the potential to increase the speed of multi-color imaging by 3-fold. Moreover, it can potentially increase the channel capacity of multi-color imaging by 3-fold. Although in this Chapter we limit our discussion to imaging three structures with one channel, μ Split-SIM shows promising potential to be extended to an even larger number of imaging channels.

6.2 Experiment methods

6.2.1 μ Split

To perform image decomposition, a small cropped (64×64 pixels) region of a larger image of a mixed (i.e., multi-label) sample is used as input. After the first convolution layer in the network, the input is downsampled by a factor of 2 (to 32×32 pixels). Before the downsampled input goes through a second convolution layer, the surroundings of the original (64×64) crop are added together with the downsampled image, and the total size of the input becomes 64×64 pixels again. Similarly, after a second convolution layer, the input is downsampled again, and a larger surrounding image is added to the model to keep the size of the input at 64×64 pixels. Figure 6.1 shows the basic structure of the deep learning model. A detailed description is provided in Ashesh et al. (2023).

There are several advantages to continually adding surrounding information as input to the model. Firstly, unlike spectral demixing, which processes each pixel independently, the morphology-based decomposition method relies on the overall structure of the image content to inform neural network’s ability to distinguish different structures. For this reason, the image information that surrounds the core image of each layer used as extra input provide additional contextual information that aids the model in recognizing subcellular structures more accurately. This approach ensures that the model can leverage the broader context present in the larger image to facilitate the identification of specific features, enhancing the accuracy of the decomposition process.

Secondly, instead of directly using a large image as input, adding increasingly larger surrounding image information after each downsampling step effectively keeps the total input size at 64×64 pixels, thereby significantly saving the working memory of the model. This approach allows the model to handle larger context information without the need for extensive memory resources, thus improving computational efficiency while maintaining high

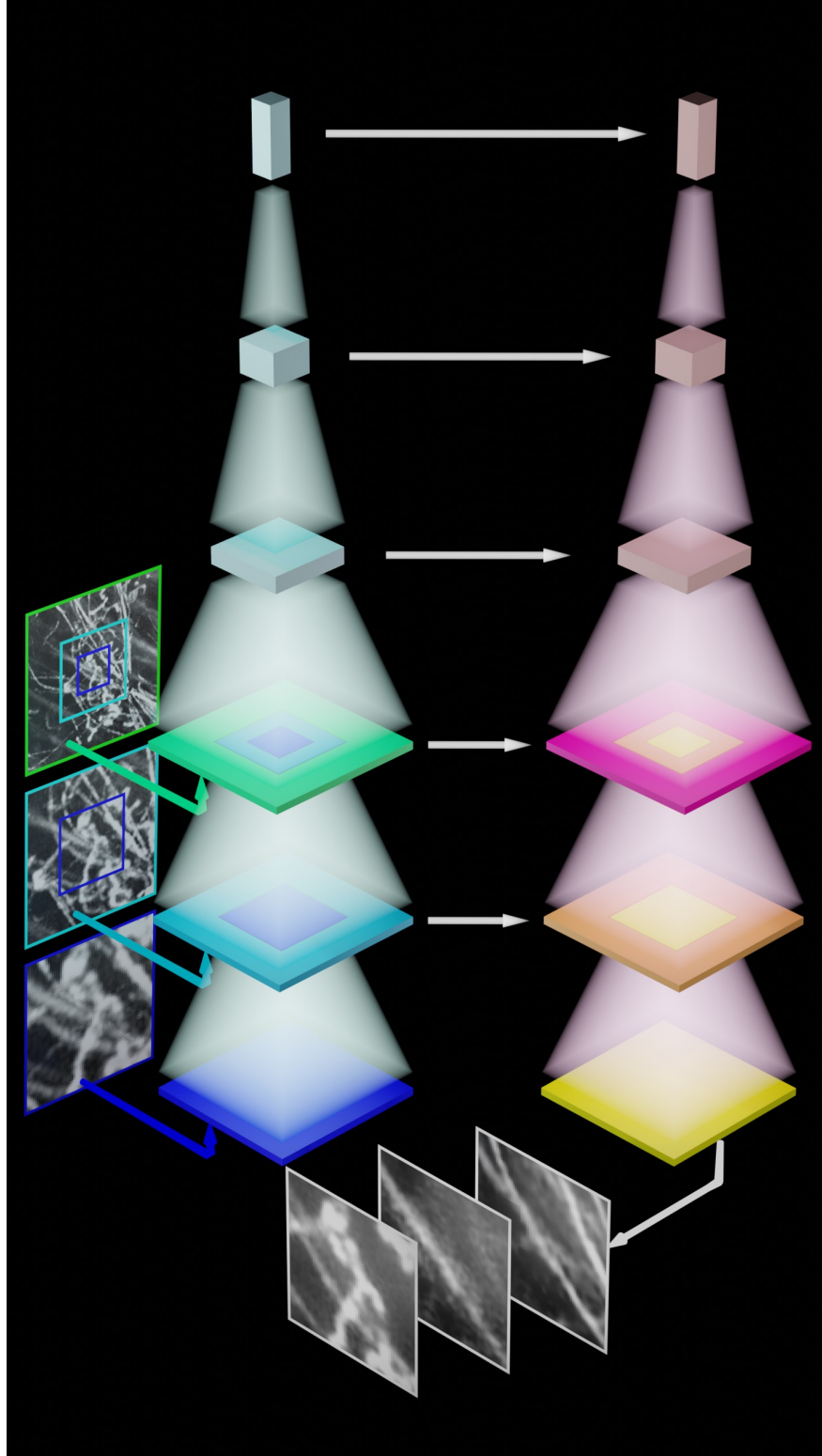


Figure 6.1: **Architecture of μ Split-SIM model.** After each downsample operation, a larger surrounding image is used as extra input to provide more contextual information to the model for structure decomposition.

performance.

Thirdly, the performance of a neural network is typically positively correlated with the number of layers. Adding surrounding image information in each layer of the model while keeping the input size constant allows for the possibility of an extra-deep neural network. For instance, a 64×64 pixel input can be maximally downsampled six times, corresponding to a six-layer neural network. On the other hand, if the input size remains constant after each downsampling, it can pass through many more layers, enabling the construction of a much deeper network. This deeper architecture can potentially enhance the model’s capacity to learn and recognize complex patterns in the data.

With the advantages described above, we will demonstrate that the μ Split model can potentially achieve memory-efficient image decomposition with high fidelity.

6.2.2 *Acquiring multi-color SIM training dataset*

To train the deep learning model, a set of large multi-color imaging dataset was acquired using the same method as described in Chapter 5 and as shown in (Gong et al. (2024)). A 3D schematic of the image acquisition light path is shown in Figure 6.2. Three different datasets were created from the large image format set of multi-color SIM images.

Data set A, 3-color 3-channel: Three different colored fluorescent labels were used (specific to mitochondria green, actin yellow, tubulin red) to label the live cells. The three labels are excited by three lasers and individually imaged by three different color filter sets. Data set A is typically used as ground truth since it provides separate images of different labels. The gray scale images of the three different channels can also be summed together to create synthesized mixed images to train the μ Split model.

Data set B, 3-color 1-channel: The same three different colored fluorescent labels were used to stain cells, but all three colors were imaged simultaneously. This was achieved by using a multi-band filter cube and turning on all three excitation lasers simultaneously

within a single camera exposure. In contrast to Data A, which synthesizes the mixed image artificially, Data set B acquires mixed images directly, providing a training data set that better matches real experimental conditions. In a real experiment, Data set B can be acquired after sequential imaging of individual channels for Data set A, allowing Data set A to naturally serve as the ground truth for the decomposed images of Data set B.

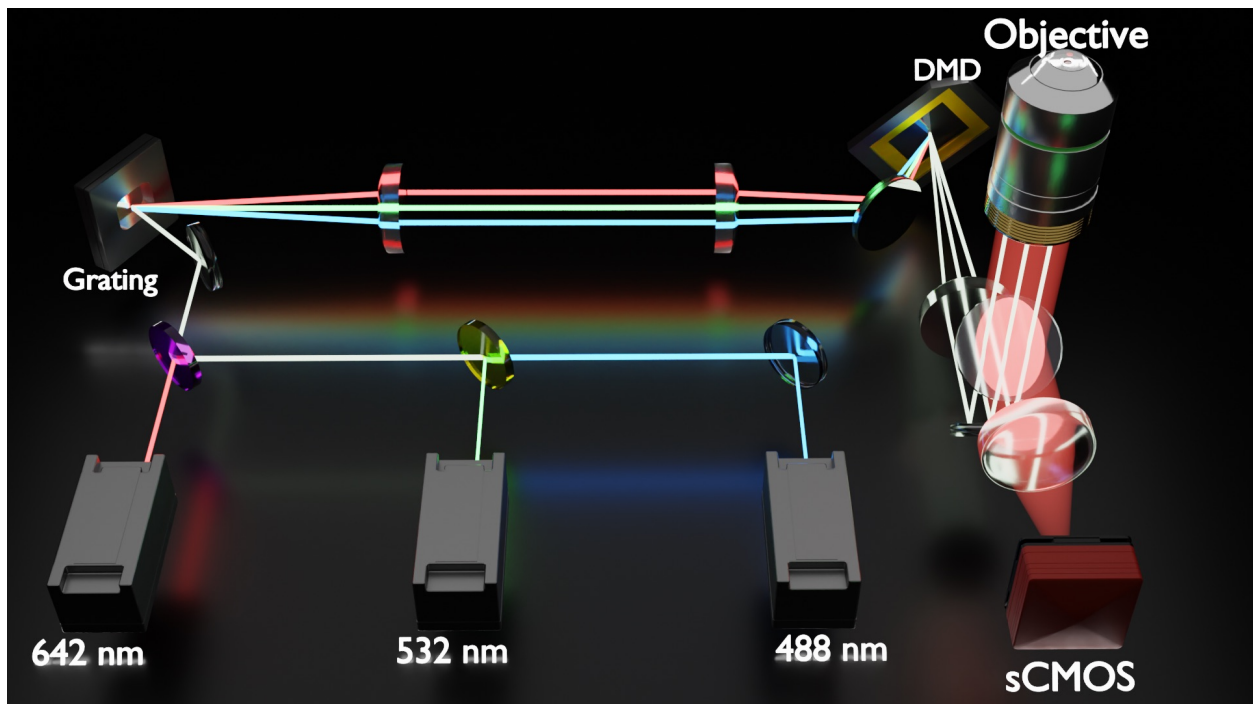


Figure 6.2: **Schematics of the data acquisition setup for the model training.** The setup is the same with the setup used in Chapter 5 for multi-color SIM imaging.

Data set C, 1-color 1-channel: Only one colored fluorescent label was used for all structures (mitochondria green, actin green, tubulin green), and all the structures were imaged simultaneously using one excitation wavelength. Compared to Data set B, Data set C is acquired using truly one color channel. Successfully decomposing Data set C is the ultimate goal for μ Split-SIM. However, it is currently challenging to acquire the ground truth data for Data set C, since only 1 label is used to label all the structures and there is no way to image the different structures separately. As a result, Data set C can only be used as a test dataset for the model, not as a training dataset.

At the time of writing this dissertation, this project is still ongoing. For this reason, we will only discuss the most basic scenario where Data set A is used as both the training data and ground truth in this Chapter. A total of 263 three-color-channel SIM images of 4096×4096 pixels each were acquired, from which 230 high-quality images were selected. Of these, 184 images were used as the training dataset, 23 images as the validation dataset, and 23 images as the test dataset. The details of the code for neural network training are shown in Appendix B at the end of this Chapter. The trend of the PSNR (eq. 6.2) during the model training is shown in Figure 6.3.

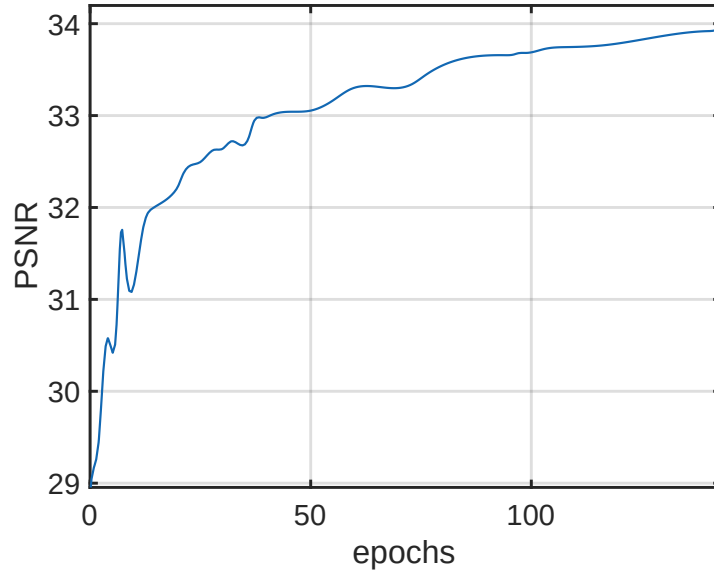


Figure 6.3: **PSNR calculated from the output of the model tested with validation dataset during the training of the model.** The PSNR keeps increasing as the training epoch increases and rea

6.3 Image decomposition result

After training with the training dataset and validation dataset, the performance of the μ Split model is tested using the test dataset. Figure 6.4 shows typical examples of the results predicted in comparison to the ground truth. Visually, the predictions from the μ Split-SIM

model match the ground truth very well.

Two metrics are used to quantify the performance of the model: Structural Similarity Index Measure (SSIM) (Wang et al. (2004, 2003)), and peak signal-to-noise ratio (PSNR). The structural similarity is a widely used method to quantify the similarity of two images from the perspective of perceived structures, rather than purely pixel values, which is particularly useful for our application.

$$SSIM(x, y) = \frac{(2\mu_x\mu_y + c_1)(2\sigma_{xy} + c_2)}{(\mu_x^2 + \mu_y^2 + c_1)(\sigma_x^2 + \sigma_y^2 + c_2)}, \quad (6.1)$$

where μ_x and μ_y are the mean pixel values of images x and y , σ_x and σ_y are the variances of the ground truth and prediction images x and y , σ_{xy} is the covariance of x and y , and c_1 and c_2 are user-defined parameters to avoid an infinitely small denominator. For our model, the SSIM measured for each channel is: 0.915 (actin), 0.894 (mitochondria), and 0.552 (tubulin). The high SSIM values for the actin and mitochondria channels suggest that the model can separate these two channels with extraordinary fidelity. The relatively lower SSIM value for the tubulin channel might result from the significantly lower signal level of the tubulin imaging channel compared to the other two channels, leading to extra difficulty for the model to separate the tubulin structures. Despite this, the prediction for the tubulin channel still shows high similarity compared to the ground truth.

The peak signal-to-noise ratio (PSNR) is also used as a metric to quantify the performance of the model, which can be expressed as:

$$PSNR = 10 \cdot \log_{10}\left(\frac{1}{MSE}\right), \quad (6.2)$$

in which MSE is the mean squared error between the ground truth x and prediction image

y. MSE is calculated as:

$$\langle (x(i, j) - y(i, j))^2 \rangle \quad (6.3)$$

PSNR is a widely adopted metric to quantify the similarity between two images from the perspective of individual pixel values, serving as a straightforward supplementary measure to indicate the performance of the model. For our model, the PSNR for each channel is: 36.82 (actin), 36.81 (mitochondria), and 29.74 (tubulin). The relatively lower PSNR for the tubulin channel is due to the low signal level of the raw training data. Despite this, the model still shows very promising performance on the tubulin channel.

6.4 Discussion and Conclusions

In this Chapter, we presented a novel deep-learning model, termed μ Split-SIM, that can decompose a single-channel image containing multiple cellular structures into a multi-channel image with all the structures well identified and therefore as a result, a multi-channel imaging task that used to require multiple light sources to excite multiple fluorescent labels sequentially can now be achieved using one light source, one label, and one imaging channel within one "snapshot." This method has the potential to significantly increase the speed of multi-channel imaging, as well as increase the channel capacity.

Compared to spectral-demixing methods, which demix images based on prior knowledge of the emission spectrum of labels, the μ Split method is purely based on the content of images and does not require any knowledge of the spectrum. This allows for much more general applications regardless of the labels used. This feature even makes it possible to use the same label (e.g., GFP) for all structures, and the model can still decompose the mixed image. Despite this advantage, we acknowledge that the model can only separate objects with morphologically distinct shapes or structures, such as actin, tubulin, nuclei, mitochondria,

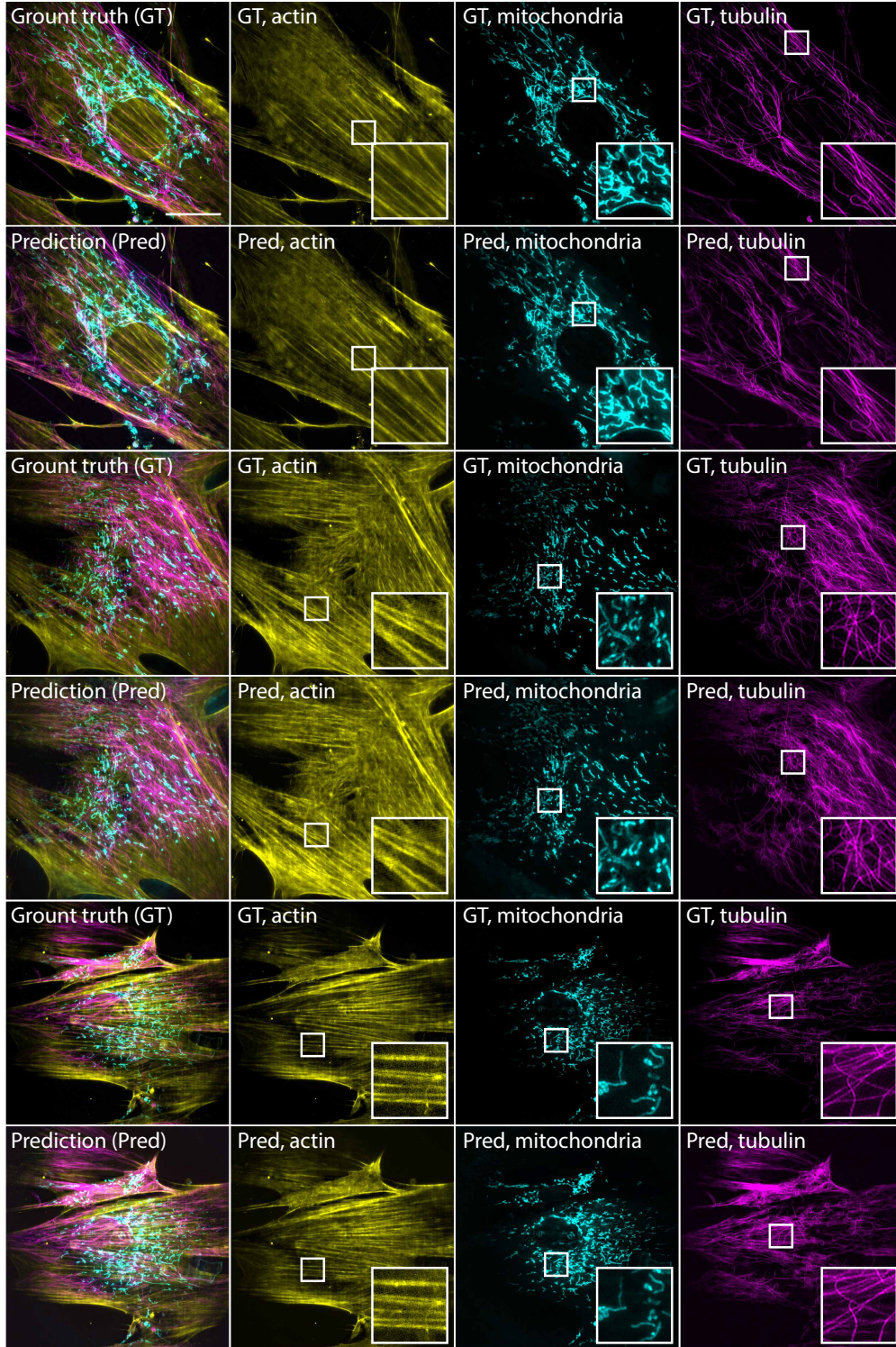


Figure 6.4: **Representative results of the channel separation tested from the test dataset.** The prediction of the decomposed three color-channels are very close to the ground truth.

membranes, etc. On the other hand, small molecules that appear as diffraction-limited points in microscope images will not be separated by our model, as they lack distinctive structural features for the model to learn.

Just like all supervised deep learning models, the μ Split-SIM model relies heavily on the amount and the quality of the training data. In this Chapter, all the images used to train the model were acquired from human fibroblast cells. However, due to the morphological variability between different cell types, the model trained on our dataset will likely need to be retrained for other types of cells.

Another source of variability comes from the imaging modality. In our work, we trained the model solely with SIM imaging data, and evidently, the performance of the model will be much less robust when used for other imaging modalities, such as confocal or light sheet microscopy. This lack of generality makes it very challenging to apply a deep learning model to different research situations. The most straightforward and effective way to address this issue is to increase the size of the training dataset to include as many diverse types of data as possible. Currently, due to limited time and resources, we restrict our discussion to the results trained with fibroblast cell images acquired via SIM imaging.

Finally, since this project is part of a collaboration led by the team that developed μ Split (Ashesh et al. (2023)), the project also involves collaboration with other labs that will contribute datasets with different imaging conditions and techniques. With all the data combined, the model will achieve much more robust performance under various research conditions. This project aims to systematically demonstrate the performance of the μ Split model in different biological research situations and will provide much more comprehensive results and analysis.

Appendix 6.A Installation of μ Split-SIM

```
1  git clone https://github.com/DZGong/uSplit.git
2  cd uSplit
3  mamba create --name usplit python=3.9
4  mamba activate usplit
5  ./install_deps.sh
6  pip install -e .
```

Appendix 6.B Code for neural network training

The model training is dependent on the actual form of the data as well as the computational platform. Here we provide an example of training our data by the Midway3 clusters service provided by computing center at UChicago. The input data SIMclean180.tif is a tif file with a size of $180frames \times 4096x \times 4096y \times 3channel$.

```
1  sinteractive --account=pi-nfschere --partition=gpu --gres=gpu:1 --cpus-per-  
   task=16 --ntasks-per-node=1 --constraint=a100 --mem-per-cpu=6000 --time  
   =36:00:00  
2  
3  module load python/anaconda-2021.05  
4  module load cuda/11.2  
5  module load cudnn/11.2  
6  source activate usplit  
7  export WANDB_MODE=disabled  
8  
9  python /home/dzgong/uSplit/usplit/scripts/run.py --workdir=/home/dzgong/  
   uSplit/train/ --mode=train --datadir=/project/nfschere/dzgong/  
   SIM_three_channel_tiff/SIMclean180.tif --config=/home/dzgong/uSplit/  
   usplit/configs/lc_paviaATN_config.py
```

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CHAPTER 7

SCI-SIM: SNAPSHOT SIM BASED ON COMPRESSIVE SENSING

7.1 Introduction

SIM has become increasingly important in biological imaging, offering a good balance between spatial and temporal resolution (Bond et al. (2022); Wu and Shroff (2018)). The “super-resolution” aspect of SIM arises from the Moiré effect: the sample is illuminated with a known periodically structured pattern of light generated with, e.g., a digital micromirror device (DMD) (Sandmeyer et al. (2019)). This high-spatial frequency pattern is used in the computational reconstruction of the sample’s high spatial frequency information from the measured (e.g., fluorescence) image; i.e., the Moiré pattern. Capturing the high spatial information of the sample requires that three 120° rotated orientations of the periodic pattern as well as a series of phase-shifted patterns in each spatial orientation be illuminated and imaged (Gustafsson (2000)). For the SIM instrument that we have constructed, this means 18 (3×6) illumination patterns and images must be recorded for SIM image reconstruction. Reasonable SNR is required, necessitating some temporal integration of the fluorescence signal on the CMOS array detector, resulting in ~ 1 sec/full SIM image. While this is adequate for studying some biological processes, it is too slow to follow phenomena like intracellular trafficking of vesicles transporting cargo such as insulin (Yi et al. (2024)). Therefore, the need to capture multiple structured illumination images to reconstruct a single super-resolution image is a significant limitation of SIM that we propose to overcome in an experimental/computational/theory collaboration.

Compressive sensing (CS) is a method that allows the reconstruction of signals from a reduced set of measurements (Calisesi et al. (2022)). In CS, one encodes random patterns into multiple images of a sample. With prior knowledge of the pattern(s), the information

of the sample can be recovered, potentially even from a single measurement. “Snapshot” Compressive Imaging (SCI), a specialized application of compressive sensing (Yuan et al. (2021)), enables the capture of high-dimensional data, such as sequences of images, in a single snapshot. Therefore, if successfully implemented, CS and SCI could decrease the image acquisition time of SIM by $>10\times$. Furthermore, both SIM and SCI utilize the same spatial light modulators, such as DMDs, for optical pattern encoding. This commonality makes the integration of the two techniques intrinsically straightforward without the need to introduce more hardware complications. Some rudimentary demonstrations combining SIM with basic compressive sensing principles have been recently reported. However, these were either just simulations (He et al. (2022)) or failed to accelerate SIM acquisition due to poor hardware and algorithm design (Ozgurun and Cetin (2020); Meiniel et al. (2017)).

The integration of Snapshot Compressive Imaging with Structured Illumination Microscopy (SCI-SIM) will overcome the speed limitations of SIM while maintaining its super-resolution capabilities. This innovative SCI-SIM approach will contribute significantly to advancing biological research for real-time super-resolution imaging of complex and dynamic biological processes in live cells.

Although there have been reports in the literature trying to decrease the number of frames required for SIM reconstruction (Ströhl and Kaminski (2017)), single-shot SIM has never been accomplished before. Our optical setup described in the preceding chapters has demonstrated high-quality SIM imaging capability, robustness against (even correction) of optical aberrations, and multi-color imaging. Therefore, we have a powerful experimental platform for realizing single-shot SCI-SIM.

7.2 Principle of SCI-SIM

The main idea of SCI-SIM is to compress the process of sequential SIM imaging by encoding the SIM patterns sampled with different random binary patterns, as shown in the flow chart

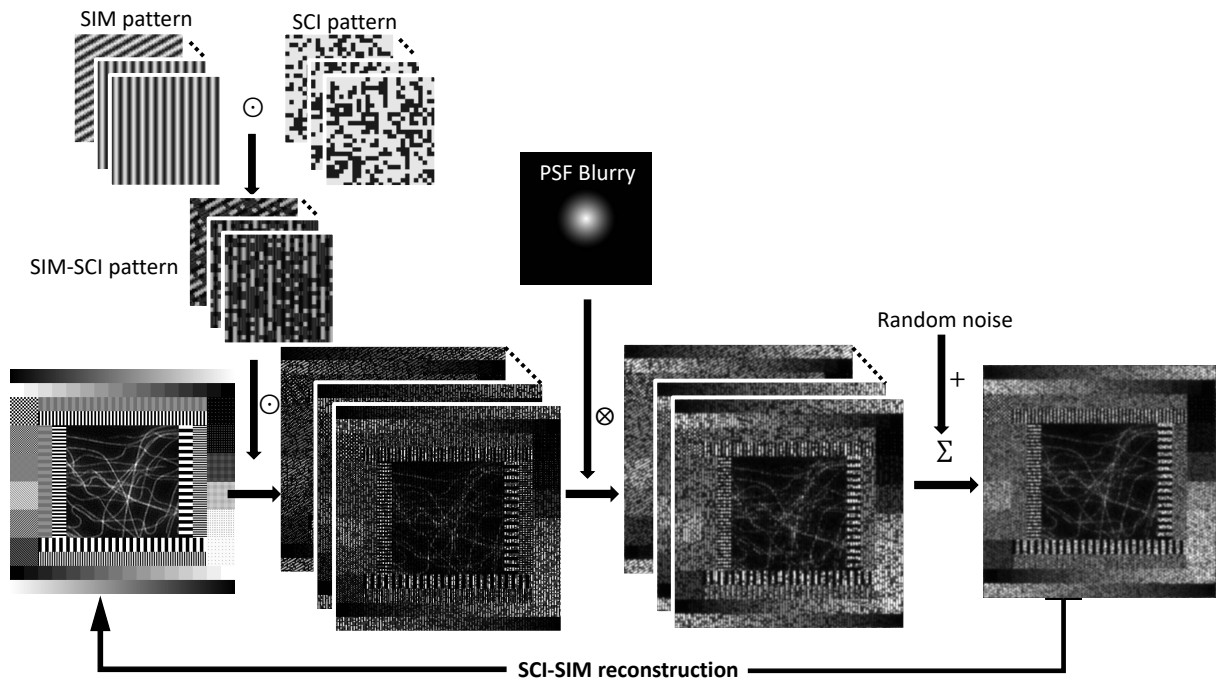


Figure 7.1: **Flow chart of SCI-SIM imaging.** The sample is illuminated by SIM-SCI patterns, which are SIM patterns encoded by SCI patterns. The SCI pattern encoding can compress the number of raw image needed to recover the high resolution information.

of Figure 7.1. This encoding process allows for the modulation of multiple raw SIM images within one camera frame. With prior knowledge of the SCI encoding pattern, a forward model can be developed to reconstruct the high-resolution image from the compressed image.

Consider that sample $s(x, y)$ is illuminated by the i_{th} SCI-SIM pattern, which is a superimposition of the i_{th} SIM illumination pattern $m_i(x, y)$ and a random binary pattern:

$$c_i(x, y) = \begin{cases} 0, & p = p_0 \\ 1, & p = 1 - p_0 \end{cases} \quad (7.1)$$

in which p_0 is the probability of the value of a pixel at (x, y) to be 0. In our case we set $p_0=0.5$; Assuming the sample is imaged under a microscopy system with a point spread function $h(\vec{r})$, the measured image will then be

$$d_i = (s \cdot m_i \cdot c_i) \otimes h \quad (7.2)$$

Now instead of using a single SCI pattern, consider the image is captured with a series of N different SCI-SIM patterns illuminating the sample sequentially in a single exposure, the measured image is going to be a summation of the images with individual SCI-SIM pattern illumination:

$$d = \sum_i^N (s \cdot m_i \cdot c_i) \otimes h \quad (7.3)$$

. The Equation 7.3 need to be re-expressed in a matrix form for the convenience of developing the reconstruction method. Firstly, the sample $s(x, y)$ with a dimension of (N_x, N_y) can be

written as a $(N_x N_y, 1)$ vector S :

$$S = \begin{bmatrix} S_{11} \\ S_{21} \\ \vdots \\ S_{N_x 1} \\ S_{12} \\ S_{22} \\ \vdots \\ S_{N_x 2} \\ \vdots \\ S_{N_x N_y} \end{bmatrix} = \begin{bmatrix} S_1 \\ S_2 \\ \vdots \\ S_{N_{xy}} \end{bmatrix} \quad (7.4)$$

In which $N_{xy} = N_x N_y$. The i_{th} illumination pattern $p_i = m_i \cdot c_i$ with a dimension of (N_x, N_y) can be written as a diagonal matrix P_i with a dimension of (N_{xy}, N_{xx}) :

$$P_i = \begin{bmatrix} P_{1,i} & 0 & 0 & \cdots & 0 & 0 \\ 0 & P_{2,i} & 0 & \cdots & 0 & 0 \\ 0 & 0 & P_{3,i} & \cdots & 0 & 0 \\ \vdots & \vdots & \vdots & \ddots & \vdots & \vdots \\ 0 & 0 & 0 & \cdots & P_{N_{xy}-1,i} & 0 \\ 0 & 0 & 0 & \cdots & 0 & P_{N_{xy},i} \end{bmatrix} \quad (7.5)$$

in which $P_{xy,i} = m_i(x, y) \cdot c_i(x, y)$. The element-wise multiplication $p_i \cdot s$ can now be re-written as matrix multiplication of $P_i S$. To include the convolution with point spread

function h , we further introduce the Fourier Transform matrix W as:

$$W = \frac{1}{\sqrt{N}} \begin{bmatrix} 1 & 1 & 1 & 1 \dots & 0 \\ 1 & \omega & \omega^2 & \omega^3 \dots & \omega^{N_x-1} \\ 1 & \omega^2 & \omega^4 & \omega^6 \dots & \omega^{2N_x-1} \\ 1 & \omega^3 & \omega^6 & \omega^9 \dots & \omega^{3N_x-1} \\ \vdots & \vdots & \vdots & \vdots & \ddots \\ 1 & \omega^{N_y-1} & \omega^{2(N_y-1)} & \omega^{3(N_y-1)} \dots & \omega^{(N_x-1)(N_y-1)} \end{bmatrix} \quad (7.6)$$

The point spread function (PSF) convolution can then be expressed as a matrix $H = W^{-1}\Lambda W$, in which Λ is the diagonal matrix form of the optical transfer function (OTF) of the microscopy system. The measured image with the i_{th} illumination pattern can then be expressed in vectorized form:

$$D_i = W^{-1}\Lambda W P_i S = H P_i S \quad (7.7)$$

The summation of D_i with a sequence of different SCI patterns can then be expressed as:

$$D = H \begin{bmatrix} P_1 & P_2 & \dots & P_N \end{bmatrix} \begin{bmatrix} I \\ I \\ \vdots \\ I \end{bmatrix} S, \quad (7.8)$$

in which I is the identity matrix with dimension of (N_{xy}, N_{xy}) .

The reconstruction of SCI-SIM can then be treated as an optimization problem by solving:

$$S = \arg \max_S ||D - AS||^2 \quad (7.9)$$

Many different algorithms have been developed to solve this kind of optimization problem,

such as ADMM (Chan et al. (2017)), GAP (Yuan (2015)), and FISTA (Beck and Teboulle (2009)). Here we adopted FISTA in combination with BM3D (Dabov et al. (2007)) as a denoiser. The algorithm can be presented as:

Algorithm 1 FISTA-BM3D for SCI-SIM reconstruction

Require: measured data D , optical transformation matrix A

$f \leftarrow A^T D$

$t \leftarrow 1$

$S \leftarrow f$

while iteration number $k < k_{max}$ **do**

$\tilde{f} \leftarrow S + \alpha A^T (D - AS)$ $\triangleright \alpha$ is a user-defined speed controlling parameter

$f \leftarrow \text{BM3D}(\tilde{f})$

$t_0 \leftarrow t$

$t \leftarrow (1 + \sqrt{1 + 4t_0^2})/2$

$S \leftarrow (t_0 - 1)/t * (f - f_0)$

end while

7.3 Simulation result

Reconstruction of simulated images is conducted to demonstrate the performance of the SCI-SIM reconstruction model. The measured data D is generated following the procedure shown in Figure 7.1. A combination of a high-resolution image of microtubules, acquired by standard SIM imaging, and a set of binary patterns is used as the original sample, S . The sample image is then superimposed with 9 SIM patterns (3 angles $\times$ 3 phases) and 9 SCI patterns to simulate the encoding process. The 9 encoded images are then blurred by convolution with the PSF (of the imaging system or a model there of) and corrupted with random noise. The final compressed image is acquired by summing all the 9 encoded images to simulate the integration of 9 patterned images within one exposure.

SCI-SIM reconstruction of the simulated input image is conducted to demonstrate the performance of Algorithm 1. Figure 7.2 shows a typical reconstruction process with $\alpha = 1$ and $k_{max} = 2000$. As can be seen, after 2000 iterations, the PSNR of the image is

significantly improved. The high spatial frequency information such as the striped pattern near the bottom of the input image is largely recovered after reconstruction.

Figure 7.3 shows a comparison of the SCI-SIM reconstructed image with the WF image and the conventional SIM reconstructed image. The SCI-SIM image shows significantly enhanced spatial resolution compared to the WF image, and the resolution is comparable to the conventional SIM image, indicating successful super-resolution reconstruction. Note that instead of using a spatial frequency shifting method like conventional SIM to generate a definite result, the SCI-SIM is purely based on iterative optimization, hence it might be difficult to determine a fixed theoretical upper limit of the reconstructed resolution. As a result, the Fourier transform of the SCI-SIM image does not showcase a clear spatial frequency boundary like WF or SIM. In some cases, the SCI-SIM shows even better reconstruction performance than conventional SIM, such as the striped patterns shown in the insets of Figure 7.3. This better reconstruction resolution in SCI imaging has also been observed in other similar work (He et al. (2022)), which is mainly due to the simple and monotonic nature of the striped pattern that allows better compression. This does not mean that the SCI-SIM can achieve generally better performance than conventional SIM. In fact, SCI-SIM will inevitably have some degradation due to the compression process, which can also be observed from the artifacts in SCI-SIM reconstructed image. However, the comparable resolution recovery achieved by the SCI-SIM with only one compressed input image makes SCI-SIM an attractive choice for high-speed super-resolution imaging without the need for sequential image acquisition.

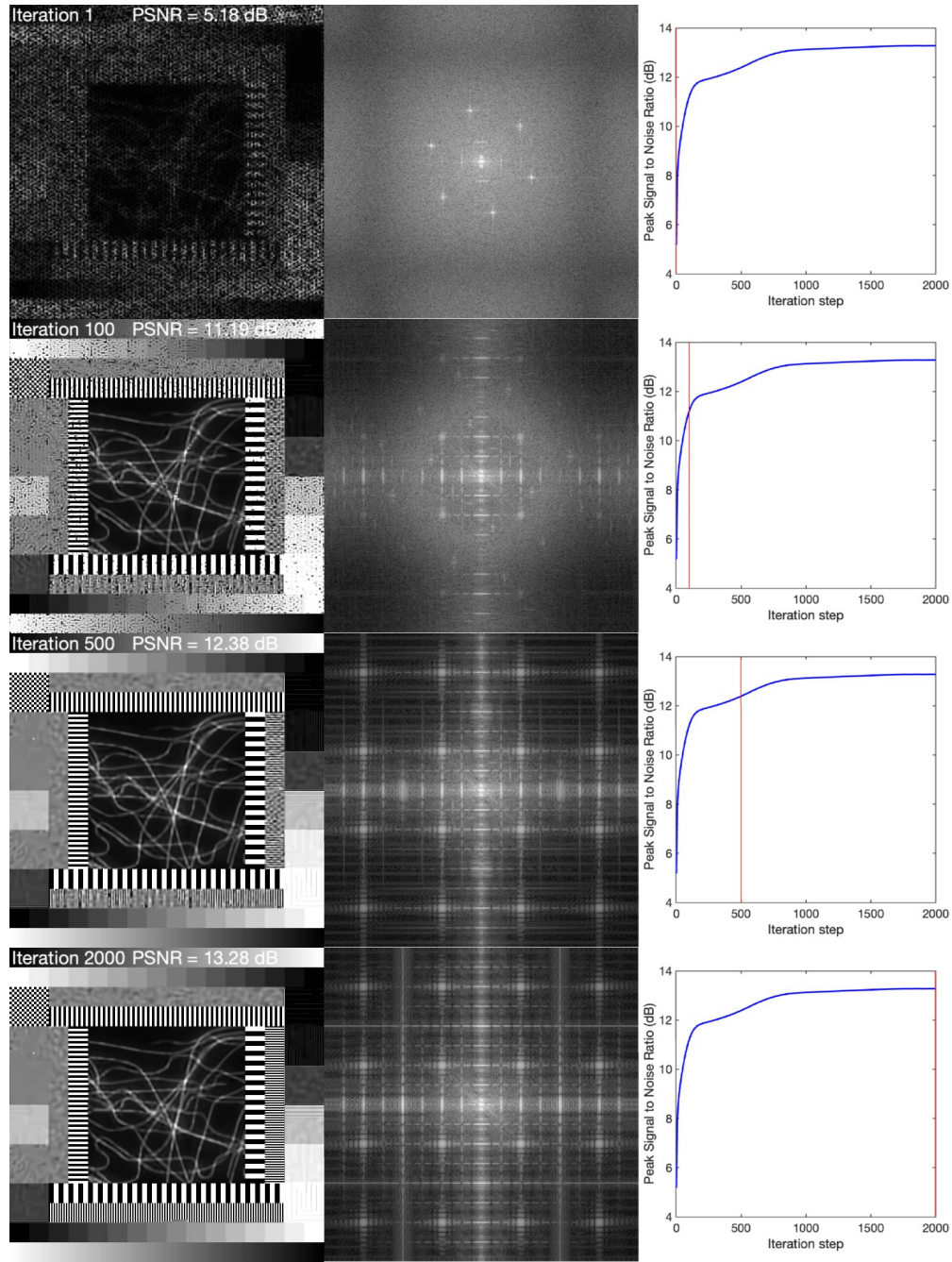


Figure 7.2: **Reconstruction result of the SCI-SIM algorithm on simulated data.** The PSNR significantly improved after ~ 2000 iterations and the high resolution information of the image was largely recovered.

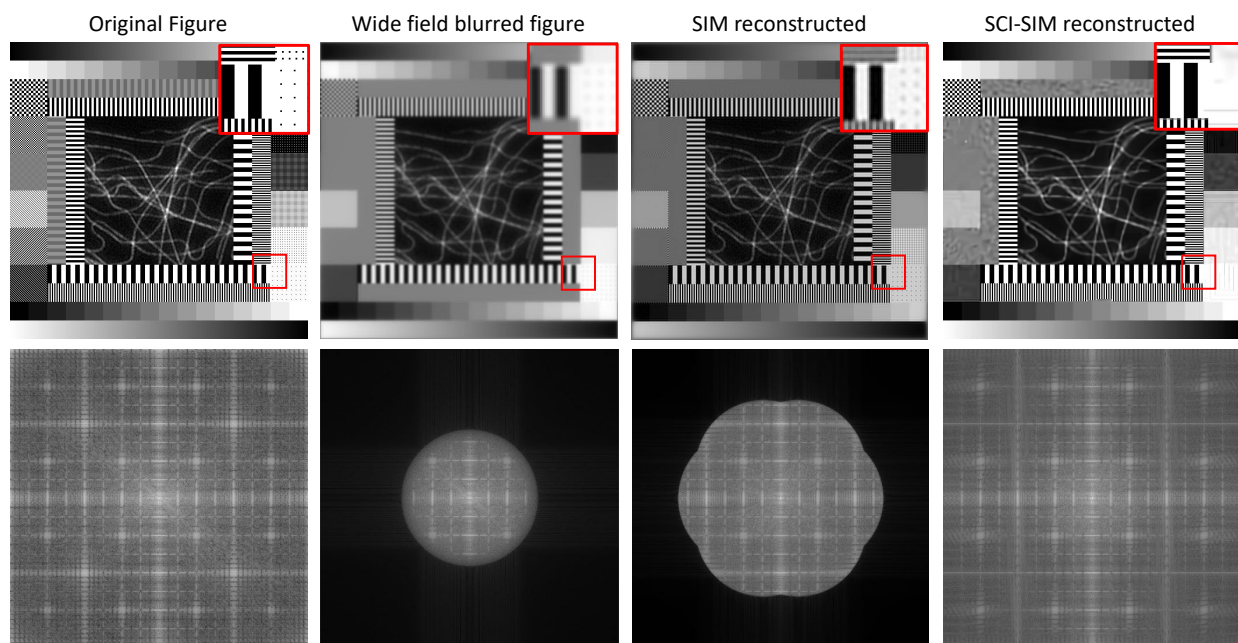


Figure 7.3: **Comparison of SCI-SIM with conventional SIM.** The resolution of the SCI-SIM is significantly higher resolution compared to wide field image, and is comparable to the standard SIM image.

7.4 Discussion and Conclusions

Ever since the invention of structured illumination microscopy, there have been ongoing efforts to decrease the number of images needed for SIM reconstruction: 9, or 7, or even 4 image based reconstructions have been demonstrated (Ströhl and Kaminski (2017); Frohn et al. (2000); Heintzmann (2003); Orieux et al. (2012)). It has been proven that once the number of patterned images is below 4, the system of equations to solve for the high-frequency information becomes underdetermined, but a proper maximum likelihood estimation can still yield resolution improvement comparable to 9-image SIM (Ströhl and Kaminski (2017)).

In this Chapter, we demonstrated a new method to decrease the number of images needed for super-resolution SIM to 1 by using the idea of snapshot compressive imaging that encodes 9 images with random binary masks and compresses them into 1 image. We developed an algorithm to perform the SCI-SIM reconstruction and demonstrated through simulation that it can achieve resolution enhancement comparable to conventional SIM. This method can significantly accelerate SIM imaging and potentially allows for super-resolution imaging with only 1 exposure needed.

We have not yet experimentally demonstrated the SCI-SIM. The SCI binary masks can be straightforwardly applied in a DMD-based SIM setup. Theoretically, one just needs to change the patterns displayed by the DMD from a standard set of SIM patterns to superimpositions of SIM patterns and SCI patterns, and display all the patterns within one camera exposure, as shown in Figure 7.4. However, there are several practical caveats that may require significant engineering effort to address.

Firstly, the SIM patterns need to maintain high quality after being projected onto the sample. In simulation, we assume an ideal optical system with no aberration. However, as we have shown in the TACO system in Chapter 3, the structured illumination patterns can suffer from local aberrations when passing through heterogeneous samples, causing significant distortions to the patterns and leading to failure of accurate SIM. Although the TACO system

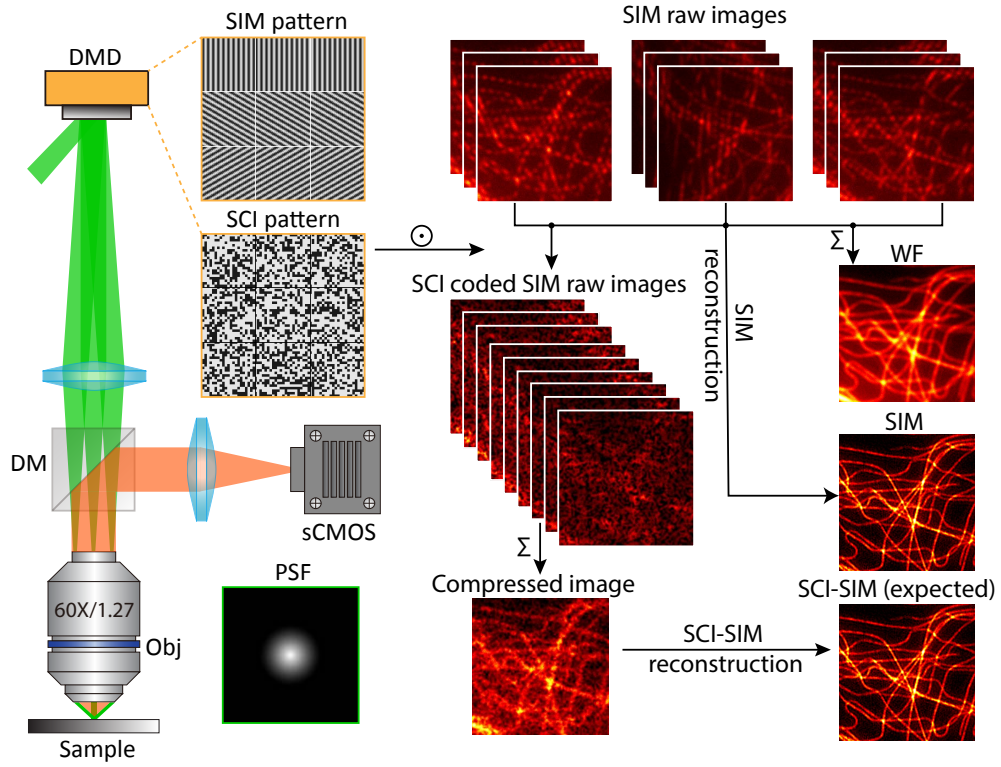


Figure 7.4: **Experimental setup for the realization of SCI-SIM.** The same setup used for the DMD-SIM in Chapter 2 can be used for achieving SCI-SIM, since the DMD can display the superimposition of both SIM pattern and SCI pattern.

can be used to correct the aberration. However, the SCI-SIM patterns are random and much more complicated than the sinusoidal SIM patterns, so defining a proper metric to evaluate the quality of the pattern may be difficult.

Secondly, precise knowledge of the illumination patterns is needed for reconstruction. This is not an issue in simulation since the patterns are nearly perfectly equivalent to the binary matrices defined by the user. In a real experiment, however, the patterns that actually illuminate the sample are very likely not the same as the binary images uploaded to the DMD, due to factors such as rotation, translation, cropping of the field of view, modulation contrast, pixelation, and aberrations caused by the imperfect imaging conditions of the light path. Consequently, before actual SCI-SIM image acquisition, a calibration process to precisely determine the pattern will be necessary, and designing a robust calibration method could be challenging.

Thirdly, a higher image speed always leads to a lower SNR, and the SNR will be even lower when using SCI-SIM illumination, since $\sim 3/4$ of the DMD pixels are turned off to display the patterns. A single image frame may be enough to acquire a WF image with acceptable quality, yet it may not be enough to acquire enough information of the 9 patterns for a high quality reconstruction. Although the exposure time can always be increased 9-fold to restore the SNR, the speed will then correspondingly decrease by 9-fold, making it meaningless to do SCI-SIM. For this reason, it may be challenging to experimentally demonstrate the speed advantage of SCI-SIM with acceptable image quality.

Although the realization in real experiments could be challenging, the prospect of success is exciting. Since the idea of SCI-SIM has been proven in theory and in simulation, I believe it is just a matter of time to solve all the experimental engineering problems. Once this snapshot imaging capability is truly achieved, it could significantly expand the application of super-resolution imaging in the study of high-speed dynamics of biological processes.

Appendix 7.A Code for SCI-SIM reconstruction

```
1 clearvars
2 clc
3
4 addpath(genpath( './algorithms' )); % algorithms
5 addpath(genpath( './packages' )); % packages
6 addpath(genpath( './utils' )); % utilities
7
8 datasetdir = './dataset/simdata/benchmark'; % benchmark simulation dataset
9 resultdir = './results'; % results
10
11 dataname = 'test2'; % [default] data name
12 datapath = sprintf( '%s/%s.mat', datasetdir, dataname );
13 load( datapath, 'meas', 'mask_sim', 'mask_cssim', 'mask_cs', 'OTFo', 'orig', 'DIOtnoisy
    ' )
14
15 A = @(z) A_xy_CSSIM( z, mask_cssim, OTFo );
16 At = @(z) At_xy_CSSIM( z, mask_cssim, OTFo );
17
18 denoiser = 'TV'; % video denoiser
19 alpha = 1;
20 maxiter = 3000; % maximum number of iteration
21 tvweight = 0.07; % weight for TV denoising
22 tviter = 10; % number of iteration for TV denoising
23
24 g=meas;
```

```

25 f = At(g); % start point (initialization of iteration)
26
27 raw=Tiff('testpat.tiff');
28 I=Tiff('reconstruct.tiff','w');
29
30 k=1;
31 t=1;
32 y=f;
33 %% IST
34
35 for iter = 1:maxiter
36     setTag(I, 'Photometric', Tiff.Photometric.MinIsBlack);
37     setTag(I, 'Compression', Tiff.Compression.None);
38     setTag(I, 'BitsPerSample', 32);
39     setTag(I, 'SamplesPerPixel', getTag(raw, 'SamplesPerPixel'));
40     setTag(I, 'SampleFormat', Tiff.SampleFormat.IEEEFP)
41     setTag(I, 'ImageLength', 512);
42     setTag(I, 'ImageWidth', 512);
43     setTag(I, 'PlanarConfiguration', getTag(raw, 'PlanarConfiguration'));
44     write(I, single(f))
45     I.writeDirectory;
46
47     f0=f;
48     ftilde=y+alpha*At(g-A(y));
49     [~,f] = BM3D(1, ftilde, 1);
50

```

```

51     t0=t;
52     t=(1+sqrt(1+4*t0^2))/2;
53     y=f+(t0-1)/t*(f-f0);
54
55     psnrall(k) = psnr(mean(f,3),double(orig)); % record all psnr
56     fprintf(' CSSIM-IST iteration % 4d, PSNR %2.2f dB.\n',...
57             k,psnrall(k));
58     k = k+1;
59
60 end
61 % close(I)
62 % close(raw)
63 subplot(1,3,1)
64 CS_final=mean(f,3);
65 imshow(CS_final,[])
66
67 subplot(1,3,2)
68 fft_final=abs(fftshift(fft2(CS_final))).^0.1;
69 imshow(fft_final,[])
70
71 subplot(1,3,3)
72 plot(psnrall)
73
74 function y = A_xy_CSSIM(z,mask_cssim,otf)
75 % z2=mask_sim.*z;
76 z2=z;

```

```

77 z3=mask_cssim.*z2;
78 y=sum(real(ifft2( fft2(z3).*fftshift(otf))),3)/3;
79
80 end
81
82 function y = At_xy_CSSIM(z,mask_cssim,otf)
83 z1=real(fft2( ifft2(z/3).*fftshift(otf)));
84 y=sum(z1.*mask_cssim,3);
85 % y=z1.*mask_cssim;
86
87 end

```

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CHAPTER 8

DISCUSSION AND OUTLOOK

8.1 Introduction

Optical microscopy imaging has served as a major workhorse in biological research and underpins a wide variety of discoveries (Zhang et al. (2023)). As research advances, high-dimensional imaging with 3D, multi-color, and time-series information is becoming increasingly important.

My Ph.D. research has centered on the development of novel snapshot multi-dimensional structured illumination microscopy methods with multiple innovations. In Chapter 2, I presented the basic framework of the DMD-based SIM that can achieve super-resolution imaging beyond the diffraction limit. In Chapter 3, I presented a novel aberration correction system, termed tandem aberration correction, TACO, to ensure high-quality SIM imaging even through aberrating media. In Chapter 4, I demonstrated a combination of multi-plane imaging with SIM, termed MS-SIM, to achieve simultaneous imaging of multiple focal planes in a 3D volumetric sample. In Chapter 5, I presented a novel system, termed gDMD-SIM, that can achieve easily scalable multi-color imaging by compensating for the dispersion effect of the DMD. In Chapter 6, I demonstrated the implementation of a novel deep learning model, μ Split-SIM, to convert a single-channel mixed image into virtual multi-channel imaging. In Chapter 7, I proposed a method to achieve single-shot SIM imaging, termed SCI-SIM, using snapshot compressive sensing. The innovations presented in this dissertation allow for snapshot 3D imaging by TACO and MS-SIM, snapshot multi-color imaging by gDMD-SIM and μ Split, and snapshot time-series imaging by SCI-SIM. Hopefully, in the future, all these techniques can be combined together so that one "snapshot" can capture all the high-dimensional data, needed for rapid volumetric super-resolution imaging.

Despite the promising performance of the presented work for acquiring multi-dimensional

imaging, I am aware that my work is nowhere close to an ideal solution that can be readily applied to biological research. Multiple issues still exist, such as the long time required to implement aberration correction in TACO, the fluorescence signal loss in MS-SIM, the low excitation energy efficiency in gDMD-SIM, and the limited generality of μ Split. All these factors may prevent these techniques from being useful for real biological research. In fact, there has always been a significant gap between what is developed in an instrumentation lab and what biologists would really like to use for their daily research (Diederich et al. (2022)). Furthermore, although the techniques presented here aspects of achieve high-dimensional imaging, they are still largely limited to several hundred nanometers in depth, a few colors, and a few milliseconds per frame. We have to ask, what are the physical limitations that prevent us from achieving much thicker imaging, a much larger number of color channels, and much faster imaging frame rates? In this Chapter, I would like to discuss an outlook for the future of microscopy imaging applications in real biological research and some of the most promising solutions to achieve real large-scale multi-dimensional imaging.

8.2 Isotropic Resolution and Large-Scale Volumetric Imaging

Optical microscopy imaging has been widely applied in 3D volumetric imaging to acquire information from not only a single plane but through an entire 3D volume. A successful 3D imaging technique needs to fulfill the following two basic requirements: high enough resolution in the z-axis, and a large enough field of view in the z-axis. We can imagine an "ultimate solution" for 3D imaging that should have isotropic resolution in both the lateral and axial directions, and can image through a range of micrometers or even millimeters thick samples. These have become long-pursuing questions in the field of 3D microscopy.

Due to the diffraction limit, when light is focused through a lens, the distribution of light intensity at the focal point is much broader along the axial direction than the lateral direction (Pawley (2006)), leading to an intrinsically poorer axial resolution compared to

lateral resolution. Although techniques such as confocal microscopy can provide optical sectioning capability by limiting depth of field and also filtering out out-of-focus light, the axial resolution is still inferior to the lateral resolution. Multiple techniques based on interference methods have been developed to achieve near isotropic resolution, such as 4-pi microscopy (Hell and Stelzer (1992)) and 4-beam-interference SIM (Li et al. (2022)), yet they generally require very careful alignment and are susceptible to reconstruction artifacts, making them impractical for real biological applications.

Another direction based on multi-view imaging, such as tomographic microscopy (Richter et al. (2017)), could fundamentally solve the isotropic resolution problem. However, it is usually not feasible to rotate the position of the objective or sample to conduct tomography imaging. One way to circumvent this issue is by using multiple objectives to simultaneously image the sample from different angles. A representative technique is diSPIM (Wu et al. (2013)), which uses two orthogonally placed objectives to conduct light sheet imaging from two different views. By switching illumination and detection between the two objectives, images from two different views can be composed into an image with isotropic resolution. This method can even be extended to four views by using reflective slides (Wu et al. (2017)). Compared to other methods, these multi-view light sheet methods are fundamentally isotropic and are easy to achieve robust reconstruction, making them widely used for real biological research. The major limitation is that they can only be applied to small transparent organisms such as *C. elegans* or zebrafish due to their limited imaging depth.

To increase the imaging depth of optical microscopy, the most widely used method is multi-photon microscopy (Denk et al. (1990); So et al. (2000)), which uses longer wavelength excitation to mitigate the effects of scattering, combined with adaptive optics (Ji (2017)) for aberration correction. This approach works very well, enabling imaging through tissues as thick as millimeters (Wang and Xu (2020)). However, the limitation of this purely optical method is clear: the illumination power needs to increase exponentially to image deep tissue,

making it fundamentally impossible to image deeper without damaging the sample.

Tissue clearing and expansion techniques (Ueda et al. (2020); Glaser et al. (2022)) have been used to decrease the scattering effects of tissues so that high illumination power is not needed to penetrate the sample. Yet, these methods can only study static not live systems since the procedures will kill the samples. To achieve real large-scale volumetric imaging, a minimally invasive way to efficiently send excitation power deep into tissues is needed. One promising direction is the use of multi-mode optical fibers as micro-endoscopes to achieve arbitrarily deep tissue imaging (Ohayon et al. (2018)). Another promising direction would be using sono-activated fluorescent labels instead of opto-activated labels (Wu et al. (2019)). By taking advantage of the high penetrating depth of ultrasound waves, these labels can be used for much deeper imaging compared to purely optical-based labels.

8.3 Large scale multi-color imaging

As we discussed in Chapters 5 and 6, despite all the hardware and software developed for multi-color imaging, the number of color channels is intrinsically limited by the wide emission spectrum of fluorescent labels. Typically, a fluorescent protein has an emission spectrum as wide as 100 nm, which limits a typical imaging system to fewer than 6 color channels (Hu et al. (2018)). Although advancements in spectral demixing methods can partially resolve the overlapping spectra, they cannot break the fundamental limit of the fluorescent labels. The width of the distribution fluorescent signal is essentially the effective pixel size of a color channel. If the pixel size is too big, no matter how advanced the imaging technique is, it will not be able to resolve the information hidden within the pixels. Therefore, the ultimate problem for multi-color imaging lies in the fluorescent labels rather than the optics. Developing new fluorescent labels with finer and sharper emission distributions becomes the key to achieving multi-color imaging of a significantly larger number of imaging targets.

One of the most attractive solutions to achieve "supermultiplexed" labeling is using

stimulated Raman scattering-based labels, whose Raman peaks can be arbitrarily tuned by engineering different lengths of polyynes (Hu et al. (2018)). Compared to the intrinsically broad fluorescent emission spectrum, Raman scattering exhibits much narrower emission, allowing for around 20 distinguishable channels within a limited bandwidth, which is much greater than for conventional fluorescent labels. Compared to other inorganic labels such as laser particles (Martino et al. (2019)) or quantum dots (Medintz et al. (2005)), these Raman scattering-based labels are much more compatible with biological labeling.

Another very promising direction is to use combinations of different labels to form unique "barcodes" for each component, rather than relying on individual labels with distinct sharp spectra. One of the most successful examples of this approach is multiplexed error-robust fluorescence in situ hybridization (MERFISH) (Chen et al. (2015b)), which can image thousands of RNA species simultaneously using combinatorial labeling. Although the number of color channels is not actually increased, this approach of combinatorial labeling achieves a geometrically scaling number of barcodes, allowing imaging of an unprecedented number of different targets.

Achieving a real large number of color channels for imaging remains a very challenging and active field in bioimaging. Eric Betzig describes this as the "holy grail" of microscopy. Considering there are so many different components interacting dynamically within an organism, it is indeed a significant problem that we can currently observe only a few of them simultaneously. It would be truly mesmerizing if hundreds or even thousands of these components could be resolved at the same time in the future.

8.4 Ultrafast time series imaging

When developing a microscopy technique, optimizing for one goal often degrades the microscope's performance in another Wang et al. (2023). Speed might be the parameter that gets compromised the most. For instance, all the super-resolution techniques that require

reconstructions from multiple frames, such as SIM or especially single molecule localization methods, essentially trade off time for higher resolution. High-dimensional imaging, such as 3D imaging or multi-color imaging, typically requires sequentially imaging through multiple z-stacks or color channels, thus slowing down the imaging speed. In fact, most microscopy problems would no longer be issues if time were not a factor. Imaging fixed samples is one example where time no longer matters since the whole sample is killed and fixed to a particular structural configuration one time point. In this case, the resolution can be theoretically arbitrarily high given long enough single molecule localization imaging time (Rust et al. (2006)) or by using expansion microscopy (Chen et al. (2015a)). 3D imaging can also be easily done by tissue clearing or even by physically sectioning the sample (Ueda et al. (2020)). Large-scale multicolor imaging can also be done by using multiple rounds of immunostaining (Loulrier et al. (2014)). Clearly, life would be much easier if time didn't matter.

Unfortunately, time matters a lot in biological imaging, especially in live organism imaging, since it is critical to observe the high-speed dynamics in organisms. For instance, voltage imaging (Bowman et al. (2023)) requires kilohertz imaging speed to resolve the membrane voltage dynamics of neuron cells.

One dilemma when it comes to imaging speed is that increasing imaging speed will inevitably decrease the exposure time of each image frame, consequently decreasing the signal that can be collected. In the early stages of microscopy, when hardware was generally slow, hardware factors such as camera framerate could be the speed-limiting factor. However, with advances in hardware development, CMOS detectors can now reach frame rates of more than a kilohertz. What really limits the imaging speed now is the SNR. In other words, for modern microscopy development, increasing imaging speed is almost equivalent to increasing the SNR of the imaging system.

The most straightforward ways to increase the SNR are by either increasing the excitation power or using brighter labels. However, these approaches reach their limitations very

quickly: excitation power cannot be increased too much due to factors such as photobleaching, phototoxicity, and heating. Furthermore, common fluorescent labels have already been largely optimized and commercialized (molecular probe catalog). With the input power and fluorescent label fixed, the signal that can be collected within a certain exposure time is fixed. Therefore, the only ways to increase the SNR are to extract as much information as possible and to suppress noise as much as possible from the finite amount of signal.

To extract more information, one of the most attractive directions is to transition from measuring averaged signal intensity using traditional detectors such as sCMOS or CCD, to measuring the arrival of individual photons using single photon detectors such as SPAD (Ulku et al. (2019)). With the information of the photon arrival time, much more information can be extracted to achieve better imaging resolution (Qi and Zhang (2023)) or determine fluorescent lifetime (Datta et al. (2020)).

For noise suppression, the most promising direction is the development of novel denoising algorithms. Imaging denoising might arguably be one of the most suitable tasks for deep learning models to solve, and there have been many new models, most of which are non-supervised or self-supervised, showing impressive performance (Krull et al. (2023); Eom et al. (2023)). Despite skepticism about the robustness and generality of these models, they are likely to be increasingly used in biological research in the future.

8.5 Retrospection

In the previous sections, I discussed some of the biggest challenges in conducting high-dimensional microscopy imaging and my personal ideas on the most promising ways to address those challenges. One thing that particularly intrigues me is that none of these challenges are likely to be solved purely by optical methods. As Stephan Hell once stated, "I realized that the fluorophore states—not the modification of the propagation of light waves—are the key to breaking the diffraction barrier" (Wang (2022)).

During my PhD experience, one thing that has become increasingly clear to me is that breakthroughs in microscopy are less and less likely to come from simple optical tricks, such as phase contrast, confocal, or light-sheet microscopy. Instead, the advancement of next-generation microscopy will likely rely on interdisciplinary efforts, including the development of new biological labels, new detectors, new ways of sample preparation, and new computational algorithms.

This realization has inspired me to choose my future research direction. Instead of purely focusing on developing optical microscopy techniques with better specifications, I would like to specialize in specific biological questions, such as neuronal activity propagation or cancer metastasis. I aim to develop new imaging techniques specifically tailored to fulfill the unique needs of these fields, making the techniques widely applicable and potentially contributing to truly impactful discoveries.

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APPENDIX A

OTHER RESEARCH

A.1 Distinct sub- to super-diffuse insulin granule transport behaviors in β -cells are strongly affected by granule age

Abstract

Intracellular transport is a complex process that is difficult to describe by a single general model for motion. Here we study the transport of insulin containing vesicles, termed granules in live MIN6 cells. We characterize how the observed heterogeneity is affected by different intracellular factors by constructing a MIN6 cell line by CRISPER-CAS9 that constitutively expresses mCherry fused to insulin and is thus packaged in granules. Confocal microscopy imaging and single particle tracking of the granule transport provides long trajectories of thousands of single granule trajectories for statistical analysis. Mean Squared Displacement (MSD), angle correlation distribution and step size distribution analysis allowed identifying 5 distinct granule sub-populations of transport, from nearly immobile and subdiffusive to run-pause and superdiffusive. The subdiffusive sub-population recapitulates the subordinated random walk we reported earlier. We show the transport characteristics of the 5 sub-populations have a strong dependence on the age of insulin granules. The 5 sub-populations also reflect the effect of local microtubule and actin networks on the transport in different cellular regions. Our results provide robust metrics to clarify the heterogeneity of granule transport and demonstrate the roles of microtubule vs. actin networks with granule age since initial packaging in the golgi (Yi et al. (2024)).

A.2 Intracellular transport of insulin granules is more complex than what the subordinated random walk model allows

Abstract

The subordinated random walk (SRW) model, which suborns the fractional Brownian motion (fBm) statistic to continuous time random walk (CTRW), is a powerful descriptor of complex transport. It has been used to describe motion of single entities (e.g., vesicles) and molecules in many systems, including live cells. However, these experimental demonstrations are typically limited in (measured) trajectory length and/or quantity of trajectories. This paper provides a critical assessment. We quantitatively analyzed particle tracking data of insulin granules in live MIN6 cells, a model line of pancreatic beta cells, that we engineered to constitutively express fluorescent insulin fusion proteins. Our experimental data set consists of over 1000 trajectories of up to 2000 time steps in duration. The trajectories show distinct behaviors (up to 5 have been characterized) that we here classify into 2 categories based on the transport exponent α : super-diffusion ($\alpha > 1$) and sub-diffusion ($\alpha < 1$). We present evidence of both fBm and CTRW random walk behavior and compare results for Mean Squared Displacement (MSD), step size distribution, ageing curve, scattering and ergodicity-breaking order parameters. We find that the SBR model cannot quantitatively account for all the experimentally observed behaviors. A goal of the present study is to motivate the development of more powerful models of transport (Gong et al. (2024)).

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