

Specific Aims

Fluorescence microscopy is an extremely valuable tool in biology—by introducing a fluorescent probe into a live organism and measuring the position of the probe, researchers can watch cellular processes as they occur. An extraordinary amount of effort has been expended towards improving the spatial resolution of fluorescence microscopes, and recent breakthroughs have allowed microscopes to achieve resolutions beyond the diffraction limit, which has enabled new biological insights [1].

In addition to the position of fluorophores, the *orientation* of fluorophores can report on biological processes [2]. Single fluorophores absorb and emit light like *dipoles*—they absorb and emit polarized light anisotropically—so their orientation can be measured using polarized light microscopes. If these fluorophores are rigidly attached to molecules of interest, then the fluorophores can provide valuable information on the orientation of those molecules. Unfortunately, current techniques can only measure the transverse orientation of fluorophores in relatively thin specimens. These constraints severely limit the number of biological questions that can be answered using available orientation measurement techniques. In this work we propose the use of polarized multiview microscopes to measure the three-dimensional orientation of fluorophores in thick living specimens. We believe that this class of techniques will enable new insights in structural and functional biology.

Scientific Premise: Three-dimensional fluorescence orientation microscopy will be a valuable tool for investigating structural and functional biology in living cells. This can be achieved using novel multiview polarization-sensitive microscopes and algorithms for estimating three-dimensional molecular orientations from such datasets.

Aim 1: Develop a signal processing pipeline for analyzing and visualizing polarized multiview fluorescence microscopy data.

The raw data collected by polarized multiview fluorescence microscopes is not easily interpreted in terms of fluorophore orientations. We propose a signal processing pipeline that can efficiently reconstruct fluorophore orientations from polarized multiview fluorescence microscopy data. An essential piece of this pipeline is the spherical Fourier transform—by analyzing the data in the angular frequency domain we can reconstruct fluorescence orientations much more efficiently than existing techniques.

Aim 2: Demonstrate and verify three-dimensional fluorescence orientation microscopy using a dual-view light-sheet microscope and a light-field microscope.

We propose two complementary three-dimensional fluorescence orientation microscopes—a dual-view light-sheet microscope and a light-field microscope. The dual-view light-sheet microscope has isotropic spatial resolution and a large field of view, but it suffers from long imaging times and it delivers a large light dose to the sample. The light-field microscope has complementary strengths and weaknesses—it images quickly and delivers a small light dose, but it suffers from anisotropic spatial resolution and has a relatively small field of view.

Aim 3: Demonstrate the value of three-dimensional fluorescence orientation microscopy for live-cell biology.

Our overarching goal is to develop useful tools that will enable biological discovery. To achieve this goal we will work directly with biologists who will propose specific questions and experiments that can exploit the techniques we are proposing. We expect that these interactions will inform and redirect our work in ways that will improve the likelihood of biological discovery.

Upon completion, this project will produce a new set of validated hardware and algorithms for determining three-dimensional molecular orientation in living samples using multiview polarized light microscopy. These techniques will help improve our understanding of basic cell biology and eventually human health.

Research Strategy

Significance

A *fluorescence microscope* is an optical imaging system that can measure the position of fluorescent objects with a resolution of $1 \mu\text{m}$ or better. Fluorescence microscopes excite fluorophores in a sample using short-wavelength light and detect the longer-wavelength light that the fluorophores emit as they relax. By focusing the emitted light onto a plane detector using a series of lenses, a fluorescence microscope creates a two-dimensional image of the fluorophores in a sample.

Fluorescence microscopes image fluorescent samples which arise in three principal ways. (1) *Endogenous fluorescent samples in nature*. Many organisms express fluorescent molecules naturally. Chlorophyll, collagen, and melanin are examples of fluorescent molecules that are found in many plants and animals. (2) *Through addition of a fluorescent dye to a sample*. There are hundreds of commercially available fluorescent dyes that can be introduced into a sample. Researchers can choose a dye that will bind to a structure or report on a process that they are interested in studying. (3) *By genetically modifying an organism so that it creates fluorescent proteins*. Many organisms can be genetically modified so that a specific protein of interest is expressed together with a fluorescent protein that labels the protein of interest. These techniques can be used to image many biological processes that involve proteins.

Most fluorescence microscope models treat fluorophores as *monopole absorbers* and *monopole emitters*, but in reality most fluorophores—including the widely used fluorophore green fluorescent protein (GFP)—behave like *dipole absorbers* and *dipole emitters*. This is an important difference. Monopoles absorb and emit all polarization states of light in all directions equally (isotropically) while dipoles absorb and emit light in specific polarization states anisotropically. By sampling the anisotropic absorption and emission patterns of a fluorophore we can estimate the *dipole axis* of the fluorophore. If the fluorophore is rigidly attached to an oriented structure in a known

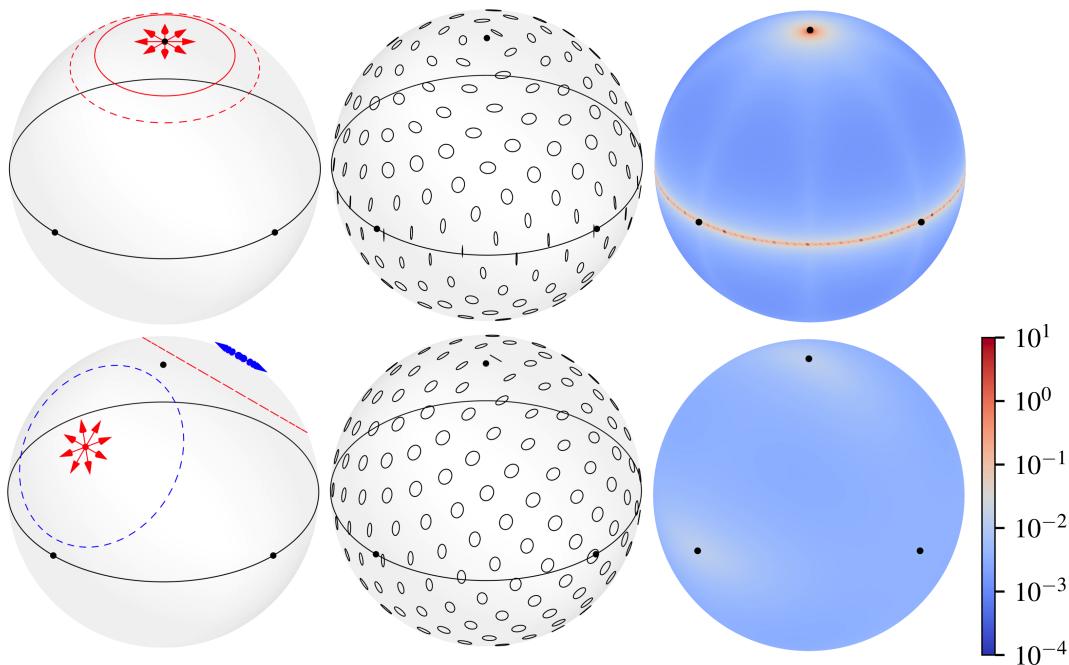


Figure 1: **Column 1:** Propagation direction and polarization of light rays—arrows indicate polarized illumination orientations, solid lines indicate illumination numerical aperture (NA), dashed lines indicate detection NA, and each color indicates a single light path. **Column 2:** Uncertainty ellipses show the relative orientation uncertainty for single molecules along different directions. **Column 3:** The solid-angle uncertainty [sr] shows the absolute orientation uncertainty in all directions. All uncertainties are for the ideal case of a fixed single dipole—motion, self-interaction (homo-FRET), and higher order radiation moments will increase the true uncertainty. **Row 1:** 0.8 NA single-view four-polarization epi-illumination microscope. **Row 2:** 0.8 NA orthogonal dual-view eight-polarization microscope. From columns 2 and 3 we can see that dual-view microscopes can estimate the orientation of fluorophores with fewer degeneracies and less uncertainty than single-view microscopes.

| Microscopy technique | Existing | | Proposed | |
|-------------------------------|-----------------------|------------------------------|---------------------------------|-----------------------|
| | Polarized single-view | Single-molecule localization | Polarized dual-view light-sheet | Polarized light-field |
| Transverse spatial resolution | ✓ | ✓✓✓ | ✓✓ | ✓ |
| Axial spatial resolution | ✗ | ✓✓✓ | ✓✓ | ✓ |
| Transverse angular resolution | ✓ | ✓ | ✓ | ✓ |
| Axial angular resolution | ✗ | ✓ | ✓ | ✓ |
| Temporal resolution | ✓ | ✗ | ✓ | ✓✓ |
| Low dose | ✓✓ | ✗ | ✓ | ✓✓ |
| Inexpensive | ✓✓ | ✓✓ | ✓ | ✓✓ |
| Large field of view | ✓ | ✓ | ✓✓ | ✓ |

Table 1: Comparison of existing and proposed fluorescence orientation microscopy techniques. ✗ = poor or non-existent, ✓ = good, ✓✓ = excellent, ✓✓✓ = best in class.

way, then we can infer the orientation of the structure from the estimated dipole axis. The monopole approximation is valid in many practical cases—when the object contains many randomly oriented fluorophores per resolution element or fluorophores that are rotating rapidly—but the complete dipole model is necessary when the object contains few fluorophores or fluorophores that are constrained to specific orientations.

Typical fluorescence microscopes are only capable of measuring the position of fluorophores, but by adding *polarizing filters* we can make a fluorescence microscope sensitive to the anisotropic absorption and emission patterns of fluorophores. If we place a polarizing filter in the excitation light path, then the light that is incident on the sample is polarized and it selectively excites fluorophores with dipole axes parallel to the light’s polarization. If we place a polarizing filter in the detection path, then we selectively detect fluorophores with dipole axes parallel to the detected polarization. By collecting images under several polarizing filter orientations and applying a reconstruction scheme, we can estimate the position and orientation of fluorophores in a sample.

We have completed a theoretical investigation of single-molecule orientation microscopy with a focus on multiview polarized light microscopes [3]. Figure 1 shows our main result—dual-view microscopes can measure the orientation of a fluorophore with fewer degeneracies and less uncertainty than single-view microscopes. These results show that three-dimensional fluorophore orientation measurements are possible with dual- and multiview microscopes.

Using polarized multiview light microscopy to measure the three dimensional orientation of fluorophores offers a new window into cell biology. Cell biologists regularly label dynamic oriented structures to watch these structures evolve—actin networks in mobile cells [4], septin networks in growing cells [5], and motor proteins for intracellular transport [6] to name a few—but current techniques limit them to investigating two-dimensional behaviors. Real cells live and behave in three dimensions, so investigating their three-dimensional structure is essential if we want to draw biological and health-related conclusions.

Innovation

Table 1 summarizes state-of-the-art techniques for measuring the orientation of fluorophores. In this section we will briefly describe each technique and its strengths and weaknesses.

Polarized single-view microscopy [5] is the simplest fluorescence orientation measurement technique. A simple single-view fluorescence microscope can be modified into a polarized single-view microscope by adding a polarizing filter to the illumination or detection path. After collecting intensity images under several polarizing filter orientations, the orientation of fluorophores can be reconstructed by applying simple image arithmetic to the set of images. Polarized single-view microscopes image quickly and gently—many live-cell biology applications already use these techniques—but they suffer from incomplete axial spatial and axial angular resolution.

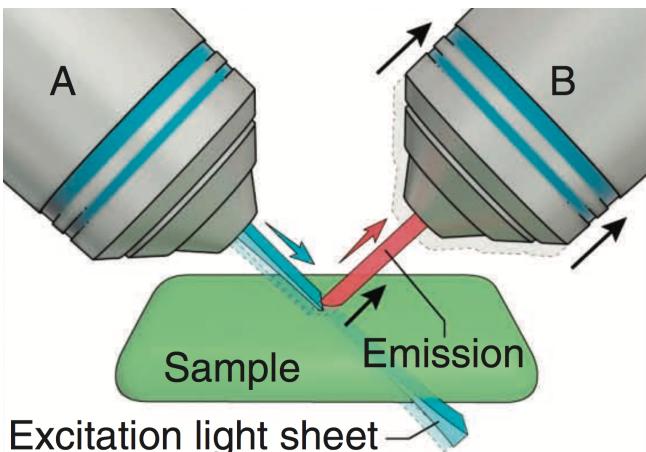


Figure 2: A dual-view light-sheet microscope excites the fluorophores in the sample with a light sheet originating from one objective, and it images the emitted light using the other objective as the light sheet sweeps through the sample. Next, the roles of the objectives are reversed and another set of images is acquired from the opposite view. We propose a dual-view light-sheet microscope outfitted with polarizing filters on each illumination arm. Figure from [7].

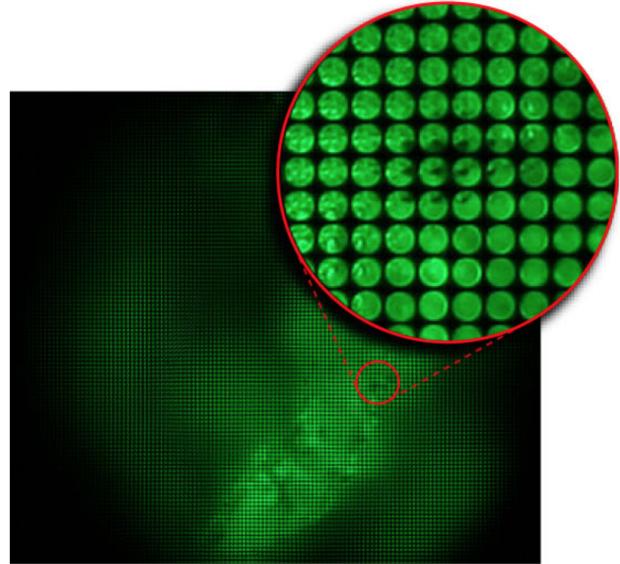


Figure 3: A light-sheet microscope is an ordinary single-view microscope with a microlens array in the usual detector plane and the detector displaced to the focal plane of the microlenses. The image collected by a light-field microscope consists of many angular views of the sample collected at once. Figure from [8].

Single-molecule localization techniques do not require any special imaging hardware—a plain fluorescence microscope can be used. Instead of special hardware, a photo-activatable fluorophore is used so that the number of excited fluorophores can be precisely controlled. By exciting and imaging fluorophores one at a time instead of all at once, the location and orientation of the fluorophores can be determined extremely precisely. Although these techniques can measure the position and orientation of fluorophores more precisely than any other light-microscopy technique, they require extremely long imaging times and large light doses. These constraints make localization techniques unsuitable for many applications in biology where fast and gentle imaging are essential for imaging live cells.

Figure 2 shows the imaging geometry for the proposed polarized dual-view light-sheet microscope. This technique uses two objectives instead of one, and it selectively excites planes of the sample to reduce phototoxicity. By using the images from both views, this technique can achieve isotropic spatial resolution, and it can measure the three-dimensional orientation of fluorophores throughout a large field of view. Although this technique does not achieve the high spatial resolution of single-molecule localization techniques, it can be applied to large-volume live-cell imaging.

Figure 3 shows an image collected by a light-field microscope. By replacing the usual detector plane with a microlens array and moving the detector to the focal plane of the microlenses, a light-field microscope captures many angular views of the sample at once. We can use these views and limited-angle tomography reconstruction techniques to determine the three-dimensional position of fluorophores throughout a sample. We propose the addition of polarizing filters to the light-field microscope to measure the three-dimensional orientation and position of the fluorophores. While the proposed light-field approach has poorer resolution than the dual-view light-sheet approach, the light-field approach is extremely fast and gentle, and it will allow us to study fast-moving processes for long periods.

Approach

Aim 1: Develop a signal processing pipeline for analyzing and visualizing polarized multiview fluorescence microscopy data.

Methods: We will use physical modeling, linear systems theory, and optimization techniques to develop an efficient signal processing pipeline for the proposed microscopes. We have completed a prototype signal processing

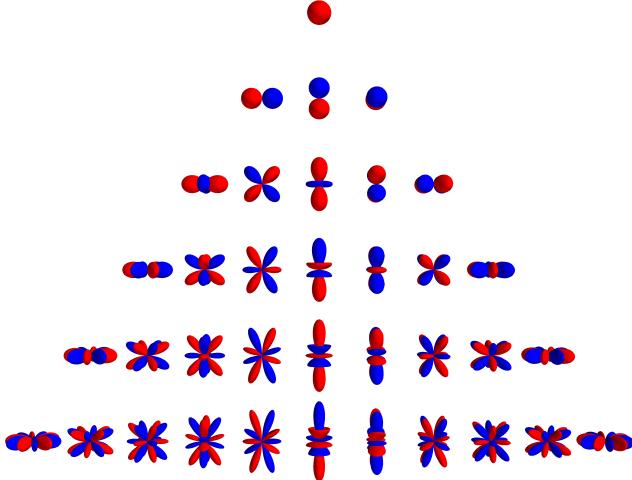


Figure 4: The spherical harmonic functions form an orthonormal basis for functions on the sphere. Red = positive, blue = negative.

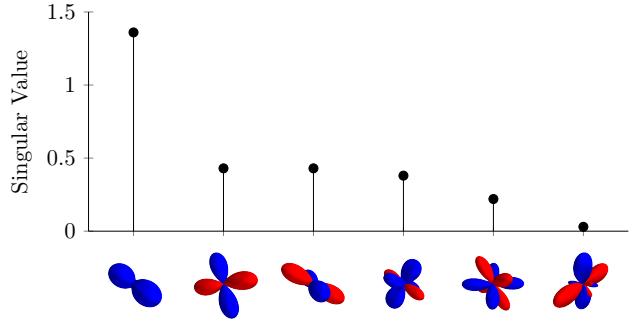


Figure 5: The angular singular value spectrum of a polarized dual-view selective plane microscope. In the same way that the modulation transfer function (MTF) characterizes the ability of an imaging system to transfer spatial harmonics, the angular singular value spectrum measures the ability of an imaging system to transfer spherical harmonics.

pipeline for reconstructing the three-dimensional orientation of fluorophores from polarized dual-view light-sheet microscope data, and we plan to improve this work by relaxing our assumptions and extending it to the light-field microscope.

To illustrate our signal processing framework, consider a small voxel that contains many non-interacting fluorophores. We define the orientation distribution function $f(\hat{s})$ as the number of fluorophores per steradian oriented in a direction \hat{s} . The goal of polarized fluorescence microscopy is to estimate $f(\hat{s})$ in many voxels throughout a three-dimensional sample.

The forward model for an N -measurement polarized fluorescence microscope is given by

$$g_i = \int_{\mathbb{S}^2} d\hat{s} h_i(\hat{s}) f(\hat{s}) \quad \text{for } i = 1, \dots, N, \quad (1)$$

where $h_i(\hat{s})$ is the kernel of the i th microscope configuration, the integral is over the sphere \mathbb{S}^2 , and g_i is the i th intensity measurement. For now we are applying this forward model to each voxel independently—we assume that there is no spatial blurring so the signal originating from each voxel is independent. By discretizing $f(\hat{s})$ and expanding $h(\hat{s})$ in terms of the spherical harmonics, we can rewrite Eq. (1) as

$$\mathbf{g} = \Psi \mathbf{B}^+ \mathbf{f}, \quad (2)$$

where $\mathbf{f} = [f(\hat{s}_1), \dots, f(\hat{s}_R)]^T$ is a vector of R samples of $f(\hat{s})$; \mathbf{B} is a matrix of the spherical harmonics evaluated at the sample points, $\mathbf{B}_{ij} = Y_j(\hat{s}_i)$ where Y_j is the j th spherical harmonic; $+$ is the Moore-Penrose pseudoinverse; Ψ is a matrix of the spherical harmonic coefficients of the kernels, $\Psi_{ij} = \int_{\mathbb{S}^2} d\hat{s} h_i(\hat{s}) Y_j(\hat{s})$; and $\mathbf{g} = [g_1, \dots, g_N]^T$ is a vector of the N intensity measurements. To reconstruct the orientation of the fluorophores in each voxel we solve the following problem

$$\mathbf{f}^* = \underset{\mathbf{f} \in \{\mathbf{e}_i\} \ i=0,\dots,R}{\operatorname{argmin}} \|\mathbf{g} - \Psi \mathbf{B}^+ \mathbf{f}\|_2^2, \quad (3)$$

where \mathbf{e}_i is a vector with a one in the i th entry and zeros elsewhere.

To our knowledge we are the first to use the spherical harmonics to analyze fluorescence orientation microscopes in the angular frequency domain—see Figures 4 and 5. These techniques allow us to use linear systems theory to understand our imaging systems, and we have already made large efficiency improvements by analyzing our microscopes using these tools.

A major limitation of our work so far is that we have assumed that the signal from each voxel is independent. In reality the signal collected by fluorescence orientation microscopes is an integral over position and orientation,

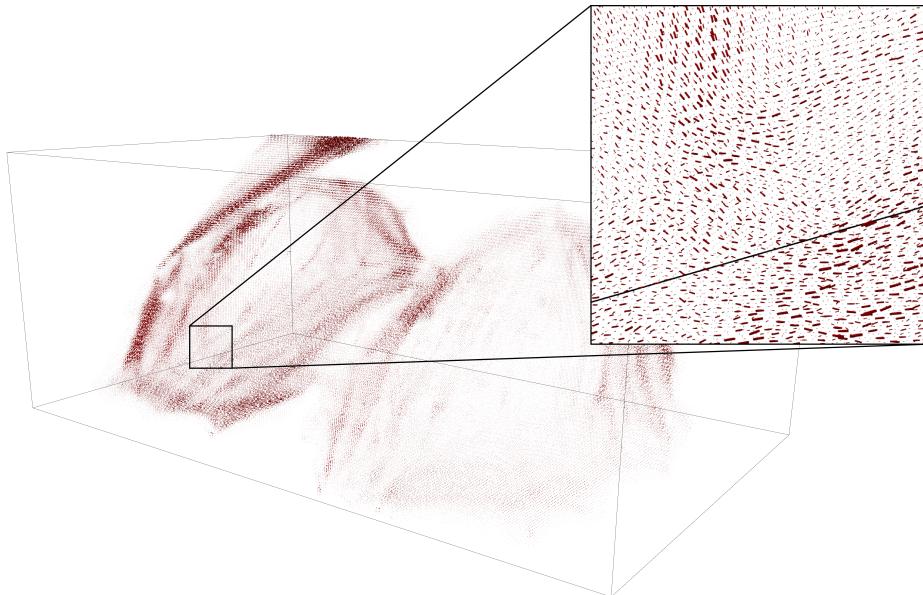


Figure 6: Orientation reconstruction of a $68 \times 108 \times 46 \mu\text{m}^3$ volume of fixed cells stained with Alexa Fluor 488 Phalloidin and imaged with an asymmetric 1.1/0.71 NA dual-view light-sheet microscope. We assign a scaled and oriented glyph to each voxel to indicate the quantity and orientation of fluorophores in that voxel. The inset shows two crossed actin fibers, and our reconstructed orientations are aligned with the long axes of these fibers as expected.

and our approximation only applies to special cases. We plan to extend our models to consider these issues, and we plan to apply joint spatio-angular restorations to the data.

Expected outcome: A set of software tools that can be used to reconstruct, visualize, and understand fluorophore orientations from data collected by a wide class of polarized multiview microscopes.

Potential complications: We anticipate computationally expensive reconstructions and visualizations as we refine our pipeline with increasingly realistic physical models. We plan to employ graphical processing units (GPUs) and cluster computing to ease the burden.

Aim 2: Demonstrate and verify three-dimensional fluorescence orientation microscopy using a dual-view selective plane microscope and a light-field microscope.

Methods: To demonstrate the dual-view selective plane microscope we will use the dual inverted selective-plane illumination microscope (diSPIM) platform developed by collaborators at the National Institutes of Health (NIH). Prototype systems are available at the NIH and the Marine Biological Laboratory (MBL). To demonstrate the light-field microscope we will use a prototype system that is available at the MBL.

To verify our systems we will use several specimens with a known coupling between fluorophore position and orientation. We are currently evaluating several samples for this purpose—PBT [poly(1,4-phenylene-2,6-benzobis-thiazole)] film, giant unilamellar vesicles [9], and actin networks.

We have started to collect preliminary data with a polarized dual-view light-sheet microscope imaging actin networks. In our first study we imaged fixed cells stained with Alexa Fluor 488 Phalloidin—a fluorescent dye that is known to attach parallel to long actin fibers in the cells. Figure 6 shows a prototype three-dimensional orientation reconstruction using a simplified model applied to real data. The results match our expectations—the reconstructed orientations align with the long axes of the actin fibers.

Expected outcomes: Clear evidence that our microscopes and reconstruction techniques can reconstruct the position and orientation of fluorophores. We will verify the position and orientation of fluorophores in a sample using existing two-dimensional techniques, and we will verify our three-dimensional techniques using this sample. We expect orientation agreement between the known sample and reconstructions within 5° for all fluorophore orientations. We also expect spatial resolution within 2 times the theoretical diffraction limit for each microscope.

Potential complications: We expect initial disagreement between our results and our known samples due to our use of simplified linear models. We expect to be able to explain these discrepancies, and we plan to refine our models if it is computationally feasible.

Aim 3: Demonstrate the value of three-dimensional fluorescence orientation microscopy for live-cell imaging.

Methods: We will collaborate with biologists at the Marine Biological Laboratory to find applications for our techniques. We have already identified several promising areas where the orientation of fluorophores in three dimensions could report useful information:

- Cell migration—the dynamics of actin networks responsible for cellular migration have been successfully studied with two-dimensional fluorescence orientation microscopy [10]. We expect that more conclusions can be drawn using three-dimensional techniques. Dr. Clare Waterman is a potential collaborator on this project.
- Cell growth—the cytoskeleton reorganizes during cell growth and division, and the reorientation of septin fibers can report these changes [4]. The proposed techniques will help biologists understand how cells grow and divide in three dimensions. Dr. Amy Gladfelter is a potential collaborator on this project.
- Cellular transport—motor protein dynamics have been studied extensively using single-molecule techniques [11]. Our proposed techniques may be able to improve on existing measurements.
- Protein-protein interactions—fluorescence resonance energy transfer (FRET) is a widely used technique for measuring nanometer-scale distances between proteins. Most FRET experiments make assumptions about the relative orientation of the fluorophores involved [12], and we expect that the proposed techniques could address these assumptions and improve the technique.

Expected outcomes: A biological research application for three-dimensional fluorescence orientation microscopy.

Potential complications: This aim depends on the previous two aims, and it requires a collaboration with a to-be-identified biologist. With our preliminary results in hand, we plan to start searching for a biological collaborator soon. During the summer the MBL acts as a hub for biologists and microscopists to test new techniques, so we expect to find a well-defined biological application in coming months.

Timeline:

| | 2018 | | | 2019 | | | | 2020 |
|---|------|----|----|------|----|----|----|------|
| | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 | Q1 |
| Aim 1: Reconstruction pipeline | | | | | | | | |
| Light-sheet reconstructions | | | | | | | | |
| Visualization | | | | | | | | |
| Light-field reconstructions | | | | | | | | |
| Refine our techniques | | | | | | | | |
| Aim 2: Experimental verification | | | | | | | | |
| Identify test specimens | | | | | | | | |
| Verify light-sheet microscope | | | | | | | | |
| Verify light-field microscope | | | | | | | | |
| Verify refinements | | | | | | | | |
| Aim 3: Live-cell demonstration | | | | | | | | |
| Identify research question | | | | | | | | |
| Investigate research question | | | | | | | | |
| Thesis | | | | | | | | |

Table 2: Proposed project timeline.

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