4Pi Single-molecule Switching Nanoscopy for Live-cell Imaging

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Abstract

Four Pi Single-molecule Switching Nanoscopy for Live-cell Imaging

Kevin Hu

2021

Fluorescence microscopy plays a critical role in cell biology due to its ability to visualize molecules of interest inside living cells with high specificity and contrast. However, diffraction of light limits spatial resolution to ~250 nm. Super-resolution microscopy bypasses this physical limit to achieve resolutions of ~10 nm, which is nearly the size of many biological macromolecules. Notably, 4Pi single-molecule switching nanoscopy achieves this resolution in all three spatial dimensions, finally making it possible to resolve specific proteins within convoluted structures like the Golgi. However, the current implementation is only compatible with dead or fixed cell samples, restricting experimental design.

To explore the complexity of life in 3-D at near molecular resolution, I have developed a 4Pi single-molecule switching nanoscope for multi-color live-cell imaging and optimized 2-color live-cell labeling with spontaneously-blinking fluorophores.
Four Pi Single-molecule Switching Nanoscopy for Live-cell Imaging

A Dissertation
Presented to the Faculty of the Graduate School
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Doctor of Philosophy

By
Kevin Hu
Dissertation Director: Joerg Bewersdorf
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Thank you. Again.
1. Introduction

1.1 Biological imaging

At each length scale, ranging from the macro down to the nano, biologists rely upon tools to observe spatial distribution and interaction. The first tool in this series is “the naked human eye”, which has resolving power on the order of a millimeter. This is sufficient to study organisms as large as blue whales and structures as small as individual organs. To study what happens on the cellular micro-scale, biologists must use light microscopes, as pioneered by Antonie van Leeuwenhoek in his documentation of the microbial world\(^1\). Inside of cells, at the nano-scale, however, this approach to seeing with visible light runs into the diffraction limit, which blocks our ability to resolve phenomena smaller than about 250 nm\(^2\).

While there are breakthroughs awaiting throughout all length scales of life, biologists have had over three centuries to explore the world with conventional microscopy. Meanwhile, nano-scale imaging techniques compatible with living specimens have only been available for a few decades\(^3\). As an example of this, eight of the last ten Nobel prizes in medicine have been awarded for discoveries involving sub-cellular molecular mechanisms\(^4\). With the current push to unravel the secrets of life at their
most basic level, there is an urgent need for techniques capable of imaging nano-scale phenomena inside of living cells.

1.2 Nanoscopy

There are several techniques capable of resolving sub-cellular processes, like endocytosis or gene regulation, that occur below the 250 nm diffraction limit for visible light. Electron microscopy, which bombards the sample with electrons instead of visible light, has been used to discover what we consider the “ground truth” in our understanding of subcellular structures\(^5\). Electron microscopy has high enough resolution that it has even been used to compute the structures of individual protein fragments\(^6\). Unfortunately, shorter-wavelength radiation also carries commensurately higher energy. This limits such techniques to studying dead samples, as the electron microscopy literally rips the sample apart during imaging\(^5\). Thus, electron microscopy is incapable of studying biological processes in living specimens.

Another method for bypassing the diffraction limit is to avoid diffraction itself. Techniques like scanning probe microscopy operate by scanning a sample with a small probe that is close enough to ignore diffraction\(^7\). For most of these techniques, resolution is a function of the probe geometry, and can approach
fractions of a nanometer\textsuperscript{3,6}. However, these methods are only capable of probing
the surface of a specimen and not its interior. To understand what happens inside
of living cells, neither electron microscopy nor scanning probe microscopy is
sufficient.

1.3 Super-resolution microscopy

Super-resolution microscopy refers to a collection of techniques that can resolve
structures below the diffraction limit using visible light in the far-field. These can be
divided into methods that improve resolution by at most a factor of two and those
with theoretically unbounded resolution. In the first group, methods such as rescan
confocal and structured illumination microscopy can resolve structures down to
~125 nm\textsuperscript{9-11}. While this is an improvement over conventional light microscopy, it is
not always sufficient and can even produce misleading results in organelles like the
endoplasmic reticulum\textsuperscript{12}.

Methods like stimulated emission depletion (STED) and single-molecule localization
microscopy (SMLM) have demonstrated resolutions an order of magnitude
better\textsuperscript{13-16}. Practically, SMLM tends to outperform STED because its performance
relies on properties of the single-molecule emitters that can be optimized while
STED resolution is more heavily dependent on the instrument and sample
conditions. As an example of its potential, SMLM has been used to resolve individual subunits within the nuclear pore complex\textsuperscript{17}.

SMLM passes the diffraction limit by leveraging a sparsity constraint and using time to separate emitters that are proximal in space. This sparsity enables the use of computational fitting to localize each molecule’s position with sub-diffraction precision. Interestingly, although the name suggests that the resolution improvement stems from the localization, it actually arises from the temporal density control or switching. There are several approaches to this density control, or “blinking”: cycling molecules through a long-lived dark state\textsuperscript{18}, photoactivation\textsuperscript{19}, photoconversion\textsuperscript{20}, photoswitching\textsuperscript{21}, photocleavage\textsuperscript{22}, and immobilization via reversible binding\textsuperscript{23} among others. Each of these methods operates using a different chemical principle, but in the context of SMLM they are functionally equivalent.

### 1.4 3-D single-molecule localization microscopy

While SMLM is powerful for improving lateral resolution, it does little to improve the axial resolution due to the anisotropy of the emitted point-spread function (PSF). Because the PSF is elongated along the optical axis, the single lateral slices
acquired are not informative about an emitter’s z-position. Despite this, biology exists in 3-space, so additional methods for improving axial resolution are critical.

3-D super-resolution in SMLM has been achieved through a number of methods that engineer the PSF to increase feature density and introduce asymmetry along the optical axis. Among the easiest to implement are the introduction of PSF-shaping phase masks or a cylindrical lens\textsuperscript{24-27}. A slight variation on this theme is the use of multiple imaging planes to take two or more measurements with known offset\textsuperscript{28}. As these techniques all function using the same principles, they have similar compromises and results and are limited to \~100 nm axial resolution. For organelles with well-defined geometries like mitochondria and chloroplasts, this is adequate\textsuperscript{29,30}. However, 100 nm axial resolution is still insufficient for organelles with complex geometries like the Golgi, which stacks and twists upon itself with thicknesses of \~30 nm\textsuperscript{31}.

To reach \~10 nm resolution in all three spatial dimensions, interferometry can be combined with SMLM\textsuperscript{32,33}. These techniques use two objectives to capture light from opposing sides of the sample. Coherently interfering the single-molecule emissions from each side creates a PSF with significantly sharper axial features\textsuperscript{34}. By acquiring at least two independent interference-phase measurements, these methods can simultaneously improve the PSF feature density and break the
symmetry to achieve ~10 nm axial resolution. More recent advances have combined this dual-objective interferometric localization with astigmatic PSF-engineering to finally visualize up to three fluorescently-labeled proteins in the Golgi in 3-D inside whole cells\textsuperscript{35,36}.

1.5 Fluorescence and multi-color imaging

Fluorescence is a powerful tool for a wide range of imaging modalities and other related assays. In the context of imaging, the primary advantage of fluorescence is that it can be used to specifically label molecules of interest with high contrast. However, this specificity is a double-edged sword; fluorescence microscopy only shows the molecules that are explicitly labeled. To alleviate this problem, microscopists often use multi-color imaging to observe how different molecules of interest co-localize. Through clever experimental design, 2-color imaging can even be used to study pseudo-infinitely many species of interest via serial use of reference stainings\textsuperscript{37}.

Multi-color fluorescence imaging methods can be split into two categories: sequential approaches, where each color is imaged in series, and simultaneous approaches, where all colors are acquired in parallel. Sequential approaches typically utilize linker chemistry that allows serial binding and unbinding\textsuperscript{38-40}. These
methods have the theoretical potential for unlimited colors, but are practically limited to roughly 40 due to epitope degradation. Because of the extended time between acquisitions, sequential methods are unsuitable for imaging live samples that can move between recordings.

Simultaneous methods, by contrast, typically arise from the ability to separate fluorescent species spectrally. This is analogous to taking red, green, and blue photos at once to merge into a full-color image as in a modern color camera. The number of colors is limited by the number of fluorescent species that can be unmixed from one another within the visible spectrum, typically no more than ten\textsuperscript{41}. One downside of imaging the full visible spectrum simultaneously is that excitation with multiple wavelengths leads to nonlinear effects like increased photobleaching and phototoxicity\textsuperscript{42}.

Fluorescence-based SMLM utilizes a variant of this, ratiometric imaging, where each single-emitter is classified by the ratio of its emissions in two or more simultaneously-acquired spectral channels\textsuperscript{43,44}. This improves crosstalk relative to conventional unmixing because each localization in SMLM corresponds to one fluorescent species while pixels in conventional microscopy contain emissions from a linear combination of fluorophores.
Selecting mutually-compatible sets of fluorophores for SMLM, however, is more complicated because the blinking energetics are chemically related to the emission spectrum and are strongly affected by off-peak excitation lasers\textsuperscript{18,22,45}. Because of these problems, along with the limited selection of viable SMLM fluorophores, simultaneous multi-color SMLM has adopted the use of combinations of far-red dyes that share a single excitation laser, have similar blinking kinetics, and require the same buffer components\textsuperscript{36,46}.

1.6 Live-cell single-molecule localization microscopy

One of the key advantages of optical super-resolution over electron microscopy is its capacity to image living samples over time. For SMLM, there are two fundamental challenges to this, namely reaching the temporal resolution required to capture dynamic activity and controlling fluorophore blinking inside living cells\textsuperscript{47}.

Improving temporal resolution in SMLM involves more than just increasing the camera frame rate; each reconstructed “frame” in a time-series must have enough localizations to resolve the structure of interest before it has time to move significantly. In turn, the rate at which localizations accrue is the product of the camera frame rate and the emitter density. Improving temporal resolution by increasing the localization rate while maintaining the sparsity constraint for individual
camera frames consequently requires faster camera speeds, higher labeling
densities, and faster-blinking emitters.

To obviate the temporal resolution requirements, initial demonstrations of live-cell
SMLM studied structures that are effectively static on the minutes-scale of standard
acquisitions. For example, the first super-resolved videos of live focal adhesions
used 30 second reconstructions to highlight movement on the
tens-of-minutes-scale. Similar era studies of MreB polymers in live bacteria
depicted structures stationary enough to not report timescales. Increased laser
power, which shortens on-times, combined with faster frame rates have enabled
temporal resolutions approaching 1 s.

While standard genetic labeling is sufficient for densely labeled structures like
clathrin-coated pits, some organelles, like the endoplasmic reticulum, do not have
individual proteins that can be labeled at high enough densities without perturbing
cell function. To address this, recent efforts have developed lipid-based labeling
methods, such as high-density, environment-sensitive (HIDE) membrane probes,
that can densely label organelles without disrupting function.

Fluorophore switching in living cells is problematic because many of the methods
currently used to control emitter sparsity are incompatible with living cells. The most
commonly used technique, dSTORM, requires cytotoxic exogenous nucleophiles and oxygen scavengers that push molecules into a long-lived dark state\textsuperscript{18}. While there have been studies using endogenous glutathione as a dSTORM nucleophile, its concentration varies widely by cellular compartment and cannot be controlled without triggering apoptosis\textsuperscript{53}. Furthermore, most SMLM protocols control emitter density via photo-activation(/conversion/switching/cleavage) with near-UV lasers that are highly cytotoxic\textsuperscript{54,55}. Other labeling schemes that do not depend on low-wavelength lasers, like DNA-PAINT, are also not viable because living cells have high nuclease activity to protect from foreign material\textsuperscript{56}. Additionally, the highest-performing organic dyes, like Alexa Fluor 647 and CF660C, are not membrane permeant.

Because of the membrane permeability requirement, most of the live-cell SMLM studies to date have used photo-controllable fluorescent proteins that can be genetically encoded\textsuperscript{20,21,57}. However, fluorescent proteins also have drawbacks when compared to their organic dye counterparts. Firstl, they generally have lower quantum yields and are less photostable, leading to worse resolution\textsuperscript{58}. Additionally, genetic encoding usually leads to variable expression and high cell-to-cell variability. While CRISPR addresses this variability, endogenous expression results in much lower labeling density\textsuperscript{17}. On top of all that, photocontrollable proteins still require near-UV illumination to control emitter density\textsuperscript{20,21,57}. 

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To remove the need for near-UV photocontrol, spontaneously-blinking fluorophores were developed. These small-molecule organic dyes combine a high-performing membrane-permeant organic dye with an intramolecular nucleophile that can reversibly bind and disrupt the conjugated π-system responsible for fluorescence\(^{59}\). Because this blinking is mediated by a bound nucleophile at fixed stoichiometry, the fraction of emissive molecules is determined by an equilibrium that can be tuned chemically. This class of probes is highly promising for live-cell SMLM with its high brightness, photostability, spontaneous blinking, and membrane permeability. However, prior to this work, there was only a single-fluorophore in this class: HMSiR.
2. Interference-based simultaneous multi-color 4Pi-SMS

The following section is being prepared for submission. It represents a new paradigm for simultaneous multi-color imaging based on phase akin to interference spectroscopy. This method is directly applicable to 4Pi-SMS and other microscope-interferometer hybrid systems.

I developed the method for interference-based fluorophore identification and performed the experimental work detailed below.
Interference-based fluorophore identification for multi-color single-molecule localization microscopy

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Abstract

We present a novel scheme for simultaneous multi-color SMLM through interference spectroscopy. This filter-free approach extracts the color-information from the interference phase shift of emitters in dual-objective SMLM. Furthermore, this approach can be simplified when the set of possible emitter species is known as in ratiometric SMLM. We show how one interference phase measurement can be sufficient to separate two or more fluorescent species.
Introduction

Fluorescence microscopy allows biologists to see specific molecules in single cells and intact organisms\textsuperscript{1}. This specificity comes at the cost of context; fluorescence-based techniques visualize only what is labeled and not the rest of the cell. To overcome this problem, microscopists use multiple fluorescent species to label targets of interest. However, there is a practical limitation on the number of fluorophores that can be imaged simultaneously due to relatively broad emission spectra and the limited range of the visible spectrum\textsuperscript{1,2}.

While there are numerous methods for sequential multi-color fluorescence imaging, none of them are suitable for live-cell experiments where it is challenging to differentiate between sample drift and biological motion\textsuperscript{3-5}. Thus, there is a persistent need for simultaneous multi-color methods that are compatible with fluorescence microscopy. We propose a conceptual advance that has the power to distinguish spectrally close fluorescent emitters through single-molecule interference.

Like dispersion, interference can also be used to capture a spectrum, as in fourier spectroscopy. Therefore, interference-based spectroscopy can be combined with single-molecule localization microscopy (SMLM) to simultaneously image multiple
fluorescent species regardless of spectrum. Such an instrument already exists in
the 4Pi-SMS, iPALM, and other such interferometric single-molecule localization
microscopes.6-8

In this work, we propose and simulate the conceptual power of interferometric
SMLM to simultaneously image and identify multiple fluorescent species based on
their phase. This innovation has the potential to vastly improve the capacity for
simultaneous multi-color super-resolution imaging.

Results

In dual-objective SMLM microscopy, interferometry is typically used only for
improved axial localization. Because interference phase depends on path length
differences between the two arms, a single-emitter’s phase depends on its relative
distance between the two opposing objectives (Fig. 2.1a). Conversely, varying the
path-length difference (sample axial position in interferometric SMLM) while
recording the phase is exactly single-molecule fourier spectroscopy.

For multi-color imaging, however, it is overkill to take a full spectrum. Because the
set of possible fluorophores is already known, one only needs to sample enough of
the spectrum to assign an unknown emitter to a probe species. One well-selected
phase measurement can be sufficient. Figure 2.1b-d illustrates this for two arbitrarily-chosen wavelengths. Even though the two start in phase, they diverge and become separable for some path-length differences ($\Delta L$). Generally, two monochromatic emitters ($\lambda_0, \lambda_1 \in \mathbb{Z}$) that start perfectly in phase ($\Delta \phi = 0$) at $\Delta L = 0$, will be perfectly out of phase ($\Delta \phi = \pi$) at

$$\Delta L = \frac{\lambda_0 \lambda_1}{2|\lambda_0 - \lambda_1|}(2k + 1) \forall k \in \mathbb{Z}.$$

If a 4Pi-SMS or iPALM instrument were configured with a pre-determined path-length difference, it would then be possible to distinguish between two emitter species based on the interference phases of their emissions.
Figure 2.1. (A) Schematic of single-molecule interferometer setup, where light is collected from two opposing sides of a sample and directed to the same detector. Interference phase depends on path-length difference $\Delta L$. (B) Example intensity traces for two arbitrarily chosen wavelengths as a function of $\Delta L$. (C) Phase of the same two wavelengths wrapping as a function of $\Delta L$. (D) Distance in phase space between the two wavelengths as a function of $\Delta L$. Note that distance cannot exceed $\pi$. 
After demonstrating how two wavelengths can be distinguished with a single phase measurement, we next investigated broad fluorescence emission spectra composed of numerous wavelengths. For this, we simulated the commonly-used SMLM dye, Alexa Fluor 647, with a typical detection band of 660-710 nm (Fig. 2.2a). Because of the finite coherence of the emission spectrum, the interference contrast decreases as the component wavelengths dephase from one another with increased path-length difference. When we compare Alexa Fluor 647 to a second commonly-used SMLM dye, CF568 (with a detection band of 580-630 nm), we can see that the two diverge in phase with increased path-length difference as expected (Fig. 2.2b).

The simulations also suggest that the monochromatic approximation used in existing interferometric localization methods is insufficient to capture the phase of a dye’s fluorescent emission spectrum (Fig. 2.2c). Additionally, interference phase is also dispersion-dependent due to wavelength-dependent path-length variation. To show this, we simulated Alexa Fluor 647 in the highly-dispersive glass, SF11 and compared the resulting phase to that of Alexa Fluor 647 in vacuum (Fig. 2.2d).

Therefore, it is important to factor in both the spectra of the fluorophores as well as the exact glasses used when calibrating a system to use this method. Additionally,
the nonlinearity in figure 2.2d suggests that using multiple glasses as in achromatic doublet lenses could enable separation of fluorophores with less loss of coherence.

Figure 2.2. (A) Normalized intensity for two common SMLM fluorophores, Alexa Fluor 647 (blue) and CF568 (orange). (B) Phase distance for Alexa Fluor 647 and CF568. (C) Phase difference between Alexa Fluor 647 and a theoretical monochromatic emitter with the same peak wavelength. (D) Phase difference between Alexa Fluor 647 with the path-length difference through SF11 glass relative to vacuum. Because path-length difference varies by wavelength for SF11, the average is used for the x-axis.
Conclusions

In summary, here we present a new paradigm for multi-color imaging through interference phase. This opens a new dimension for spectral imaging, which can be combined with conventional multi-color methods to further increase the number of fluorophores that can be imaged simultaneously.

Practically, the exact path-length difference is often dependent upon an emitter’s unknown depth in samples with finite thickness. This can be resolved with other methods for determining an emitter’s axial position that are common in SMLM, such as the use of astigmatic or double-helix PSFs. While it may seem that this negates the axial resolution enhancements of interferometric SMLM, the phase and axial position can be fitted simultaneously with an improved model function factoring in the known fluorophore species. This simultaneous fitting strategy can already be applied to improve current phase-fitting methods that treat fluorophores as monochromatic emitters, even for single-color imaging.

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References


3. Ratiometric multi-color SMLM in living cells

The following section is reprinted with permission from Tyson J, Hu K, Zheng S, Kidd P, Dadina N, Chu L, Toomre D, Bewersdorf J, and Schepartz A. Extremely Bright, Near-IR Emitting Spontaneously Blinking Fluorophores Enable Ratiometric multi-color Nanoscopy in Live Cells. ACS Cent. Sci. 2021, 7, 8, 1419-1426, DOI: 10.1021/acscentsci.1c00670 Copyright 2021 American Chemical Society. It details the rational design second spontaneously-blinking fluorophore, Yale$_{676sb}$, and its use for ratiometric 2-color imaging alongside HMSiR. The supplementary information has been omitted for brevity, but can be found online through the ACS Central Science portal.

My contribution to this work was the development of the SMLM instrument capable of ratiometric imaging of Yale$_{676sb}$ and HMSiR alongside all of the imaging and related analysis. I also conducted the single-molecule studies that were used to select Yale$_{676sb}$ from a set of candidate molecules.
Extremely Bright, Near-IR Emitting Spontaneously Blinking Fluorophores Enable Ratiometric Multicolor Nanoscopy in Live Cells

Jonathan Tyson, Kevin Hu, Shuai Zheng, Phylicia Kidd, Neville Dadina, Ling Chu, Derek Toomre, Joerg Bewersdorf, and Alanna Schepartz

ABSTRACT: New bright, photostable, emission-orthogonal fluorophores that blink without toxic additives are needed to enable multicolor, live-cell, single-molecule localization microscopy (SMLM). Here we report the design, synthesis, and biological evaluation of Yale675ab, a photostable, near-IR-emitting fluorophore that achieves these goals in the context of an exceptional quantum yield (0.59). When used alongside HMSIR, Yale675ab enables simultaneous, live-cell, two-color SMLM of two intracellular organelles (ER + mitochondria) with only a single laser and no chemical additives.

INTRODUCTION

Single-molecule localization microscopy (SMLM) is a powerful technique for visualizing intracellular architecture at the nanoscale and across large fields of view. The technique is characterized by the detection and localization of fluorescent markers that cycle rapidly between emissive (ON) and non-emissive (OFF) states. For optimal results, the sample and imaging conditions must maintain the majority of fluorescent markers in the OFF state, such that the neighboring molecules in the emissive ON state can be treated as sparse single emitters. Organic fluorophores are favored over fluorescent proteins for SMLM because they are generally brighter and more photostable and because their photophysical properties can be fine-tuned using chemistry. The challenge is that many SMLM-compatible organic fluorophores require the addition of exogenous nucleophiles, redox modulators, and/or oxygen depletion systems to switch efficiently between ON and OFF states. These additives can be cytotoxic and damage other biological samples. An additional challenge is that many established SMLM-compatible fluorophores are cell-impermeant and/or require cytotoxic high-power and/or short-wavelength lasers.

The spontaneously blinking fluorophore (SBF) hydroxy-methyl Si-rhodamine (HMSIR) (Figure 1a) reported by Urano and co-workers overcomes many of these limitations. It is cell-permeant and photostable and is believed to cycle rapidly between ON and OFF states by virtue of a pH-dependent spirocyclization reaction that occurs in the absence of chemical additives (Figure 1a). For HMSIR, the midpoint of this pH-dependent equilibrium (referred to as pK_spd) occurs at approximately pH 6.0. Thus, at pH 7.4 roughly 98% of the HMSIR molecules in solution occupy the OFF state, which enables facile detection and localization of the sparse subset of molecules that are emissive (ON). HMSIR’s cell permeability, photostability, and ability to blink in the absence of chemical additives has enabled multiple minimally invasive single-color SMLM experiments, including those that visualize organelle membrane dynamics in live cells for extended times, others that resolve the morphology of dopaminergic neurons in an intact Drosophila melanogaster adult brain, and still others that enable turn-on visualization of intracellular protein targets.

Despite these advances, there remains a need for new SBFs that effectively partner with HMSIR to enable multicolor live-cell SMLM experiments without the need for chemical additives or photoactivation. Although two green-emitting SBFs whose emission spectra are separable from HMSIR have been reported (Figure 1b), including one (HEtETFER) that can be paired with HMSIR for two-color SMLM in fixed cells, their use demands high-intensity lasers that excite at 488 and 561 nm, respectively. These light sources can induce substantial cytotoxicity as phototoxicity is especially pronounced in the blue and green spectrum. Two other previously reported SBFs are excitable in the far-red/near-IR but they are spectrally indistinguishable from

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HMSIR and therefore not suitable for two-color experiments. Although both the fluorescent protein mEos3.2 and CP550 (Figure 1d), a carboporphyrin fluorophore that reacts irreversibly with intracellular glutathione, have been paired with HMSIR for two-color, live-cell SMLM, 15,20 these experiments require an additional ~560 nm laser, which is inferior to red-light excitation for live-cell microscopy. 10,14 Furthermore, sequential multicolor imaging with multiple lasers is slow and the images are prone to sample motion artifacts. Finally, the spontaneously blinking carbophoramine, HMCRR550, which was designed using quantum calculations, has an excitation maximum at 560 nm and would likewise require multiple lasers to pair with HMSIR (650 nm excitation) for a two-color live-cell SMLM experiment.

Here we report the rational design of a new near-IR-emitting SBF that pairs effectively with HMSIR to enable simplified two-color SMLM experiments in live cells (Figure 1d). Yale676eb emits at 694 nm, the longest wavelength of any reported SBF, and possesses, to our knowledge, a higher quantum yield (0.59) than any previously reported nanoscopy-compatible Si-rhodamine (SiR) fluorophore. Yale676eb and HMSIR can be excited simultaneously with a single 642 nm laser and imaged ratiometrically for simultaneous multicolor SMLM of two distinct intracellular organelles (ER + mitochondria) in live cells.

RESULTS

New Spontaneously Blinking Fluorophores: Design Considerations. Three distinct chemical and photophysical properties are needed to ensure compatibility with HMSIR for ratiometric two-color, live-cell SMLM. The first is an emission maximum > 690 nm to ensure adequate separation from HMSIR (emission maximum = 670 nm) via ratiometric imaging. 23,31 The second is a pK_{cys} value between 5.3 and 6.0 to ensure the sparsity of emissive/ON molecules. 15,21 The third requirement is a high quantum yield; although a quantum yield > 0.2 can yield respectable SMLM images, higher values are always more desirable. 13,22 The challenge is that the quantum yields of rhodamine-based fluorophores typically decrease as the absorption and emission maxima increase (Supporting Information (SI) Figure S1). As a result, molecules that absorb and emit at higher, less cytotoxic wavelengths that are compatible with live cells are relatively dim. This correlation is reflected in the relatively low quantum yield of HMSIR (0.31) when compared to those of the green-light-emitting SBFs HMJFS26 (0.87) 16 and HE tetTFFER (0.76). 18 We therefore sought a design approach that would yield fluorophores possessing both long-wavelength emission and high quantum yield.

HMSIR_{redol}, HMSIR_{redgl} and HMSIR_{redMQ}. Previous work has demonstrated that introduction of heterocyclic indoline, 32,33 julolidine, 34 or tetrahydroquinoline 32 moieties into the core of a SiR chromophore can shift the excitation and emission maxima by up to 50 nm relative to SiR itself (Figure 2a). To evaluate whether these effects would be preserved in the context of a HMSIR core, we synthesized HMSIR, as well as the heterocyclic derivatives HMSIR_{redol}, HMSIR_{redgl} and HMSIR_{redMQ} (Figure 2b and Supporting Information S1–S4) according to a recently reported general method for Si-rhodamine fluorophore synthesis. 35 We then characterized the photophysical properties and aqueous spirocyclization equilibrium (pK_{cys}) of each new fluorophore (Figure 2b–d).

Each of the new fluorophores displayed absorption (Figure 2b) and emission (Figure 2c) maxima that were red-shifted by at least 23 nm relative to HMSIR, with the emission maxima increasing in the order HMSIR < HMSIR_{redMQ} < HMSIR_{redgl} < HMSIR_{redol}. As expected, the absorption and emission maxima of the HMSIR series were nearly identical to those of the analogous SiR variants reported previously. 12–14 The pK_{cys} of each new HMSIR analog was determined from a plot of the pH dependence of the absorption and emission maximum of the open/ON form (Figure 2d and SI Figure S2); pK_{cys} is the pH at which the concentration of the open/ON state equals that of the closed/OFF state. 15 The pK_{cys} values of HMSIR_{redol} and HMSIR_{redMQ} were 6.4 and 6.9, respectively, both significantly higher than the value for HMSIR (6.0). The pK_{cys} value of HMSIR_{redgl} (pK_{cys} = 9.0) was shifted even more dramatically, presumably because the additional electron-donating alkyl groups disfavor cyclization.

A related previously reported rhodamine analog with julolidine
groups also displayed a high pK_{cycl} value.\textsuperscript{15} The absorbance vs pH curves for HMSIR_{nded}, HMSIR_{pial}, and HMSIR_{THQ} are sigmoidal, whereas that of HMSIR is bell-shaped due to cyclization of the protonated fluorophore at low pH; this protonation is disfavored when the exocyclic amine is constrained by a five- or six-membered ring.

The final criterion needed to ensure compatibility with HMSIR for ratiometric two-color, live-cell SMLM is a high quantum yield. The quantum yields measured for HMSIR_{nded}, HMSIR_{pial}, and HMSIR_{THQ} also paralleled the values for the analogous SiR variants; the quantum yield of HMSIR_{nded} like SiR700, was low (0.13), whereas those of HMSIR_{pial} and HMSIR_{THQ} (0.43 and 0.38, respectively) were comparable to that of HMSIR (0.31) (SI Figure S3).

These data indicate that neither HMSIR_{THQ} HMSIR_{pial} nor HMSIR_{nded} possess the characteristics necessary to partner with HMSIR for two-color SMS nanoscopy. Although all three fluorophores exhibit emission maxima that are shifted by at least 23 nm from that of HMSIR, and HMSIR_{pial} and HMSIR_{THQ} display acceptable quantum yields (0.43 and 0.38), none feature pK_{cycl} values low enough to prevent significant multimeter artifacts at physiological pH. In each case, chemical modifications are needed to increase the electrophilicity of the xanthene core, favor spirocyclization, and decrease pK_{cycl}. Ideally, these modifications should also increase quantum yield to increase brightness and resolution, but as outlined below, this goal is complicated by the complex interplay between quantum yield, emission maximum, and pK_{cycl}.

**Interplay between Quantum Yield, Emission Maximum, and pK_{cycl}**. The quantum yields of rhodamine fluorophores are limited by a nonradiative decay process known as twisted intramolecular charge transfer (TICT).\textsuperscript{27-30} TICT involves the excited-state transfer of an electron from the exocyclic nitrogen of the fluorophore to the neighboring carbon \pi system with concomitant twisting of the C_{aryl}-N bond; the charge-separated state subsequently decays to the ground state without emission of a photon. Processes that decrease the propensity for C_{aryl}-N bond rotation increase quantum yield. For example, the quantum yields of rhodamine B and tetramethyl rhodamine (TMR) are higher in viscous solvents\textsuperscript{37} and at low temperature where C_{aryl}-N bond rotation is inhibited.\textsuperscript{37,40} Indeed, the modestly increased quantum yields of HMSIR_{pial} (0.43) and HMSIR_{THQ} (0.38) relative to HMSIR (0.31) can be ascribed to restricted C_{aryl}-N bond rotation,\textsuperscript{34} although these effects appear to be less dramatic in the SiR series than with conventional rhodamines: rhodamine 101, the rhodamine analog of HMSIR_{pial} displays a near-perfect quantum yield of 0.99.\textsuperscript{40}

TICT is also inhibited in fluorophores in which the ionization potential (IP) of the exocyclic nitrogen is increased by electron-withdrawing groups (EWGs).\textsuperscript{16,36,41} Addition of EWGs to a fluorophore core also decreases pK_{cycl} by lowering the energy of the fluorophore’s lowest unoccupied molecular
Figure 3. Structures and photophysical properties ($\lambda_{	ext{abs}}$, $\lambda_{	ext{em}}$, and $\Phi$) of (a) HMSIR and HMSIR$_{2\text{-F}}$Et, and (b) HMSIR$_{THQ}$, Yale$_{766ab}$, and Cal$_{664ab}$. Normalized (c) absorption and (d) emission spectra of HMSIR, HMSIR$_{2\text{-F}}$Et, HMSIR$_{4\text{-F}}$, and HMSIR$_{T}$ in 0.2 M sodium phosphate (pH = 4.5 for HMSIR; pH = 2.0 for HMSIR$_{2\text{-F}}$Et, HMSIR$_{4\text{-F}}$, and HMSIR$_{T}$). (e) pH-dependent spirocyclization equilibria. Normalized absorption of open form of 2 $\mu$M HMSIR, HMSIR$_{2\text{-F}}$Et, HMSIR$_{T}$, Yale$_{676}$, and Cal$_{664}$ as a function of pH in 0.2 M sodium phosphate buffer at room temperature.

orbital (LUMO). However, the addition of EWGs typically induces moderate to large decreases in excitation and emission wavelength maxima. For example, an EWG-containing fluorophore reported by Lv et al. possesses an exceptional quantum yield (0.66) but is blue-shifted by ~20 nm relative to HMSIR (0.53). This suggests that different combinations of restricted aryl-N bond rotation with an EWG would simultaneously increase $pK_{\text{Ryd}}$ and increase quantum yield by inhibiting TICT. If these changes were introduced into the HMIIR$_{THQ}$ scaffold, even a moderate decrease in excitation and emission maxima would not jeopardize the emission shift needed to remain orthogonal to HMIIR. HMIIR$_{THQ}$ was preferred as a starting point because its $pK_{\text{Ryd}}$ value (6.9) and quantum yield (0.38) are both close to those of HMIIR in contrast to HMIIR$_{T}$ at which quantum yield is low (0.13), or HMIIR$_{4\text{-F}}$ab whose $pK_{\text{Ryd}}$ is very high (9.0).

Design of the Bright, Near-IR-Emitting SBF, Yale$_{676ab}$. To test this hypothesis, we synthesized Yale$_{676ab}$, a variant of HMIIR$_{THQ}$, which has replaced symmetrically by mono-fluorinated N-ethyl groups (Figure 3 and S1 Scheme S5). As predicted, Yale$_{676ab}$ was characterized by a 10-fold more favorable spirocyclization equilibrium than HMIIR$_{THQ}$ ($pK_{\text{Ryd}} = 5.9$ vs 6.9) and a greatly improved quantum yield (0.59 vs 0.38) (S1 Figure S3). Interestingly, Yale$_{676ab}$ exhibited absorption and emission maxima that are both virtually identical to those of HMIIR$_{THQ}$. Addition of a stronger difluorinated N-ethyl group to generate Cal$_{664ab}$ resulted in a further increase in quantum yield to 0.74 (S1 Figure S3) but, in this case, led to an emission $\lambda_{\text{em}}$ that was too close to that of HMIIR (667 nm vs 677 nm) to support two-color ratiometric imaging. The photophysical properties associated with Yale$_{676ab}$ suggest that it should be an ideal partner for HMIIR: an emission maximum $> 690$ nm, a $pK_{\text{Ryd}}$ value between 5.3 and 6.0, and a high quantum yield. The quantum yield of Yale$_{676}$ (0.59) is, to our knowledge, higher than any Si-rhodamine derivative prepared and utilized for fluorescence nanoscopy.

To deconvolute the effects of aryl-N bond rotation and the monofluoror electron-withdrawing group, we also prepared HMIIR$_{2\text{-F}}$Et, which carries the same monofluorinated N-ethyl groups but allows aryl-N bond rotation (S1 Scheme S7). HMIIR$_{2\text{-F}}$Et was characterized by a minimal change in absorption and emission $\lambda_{\text{em}}$ relative to HMIIR; however, it displayed a 10-fold more favorable spirocyclization equilibrium than HMIIR ($pK_{\text{Ryd}} = 5.0$ vs 6.0), a value too low for efficient blinking at physiological pH of 7.4. Its improvement in quantum yield was more modest relative to Yale$_{676ab}$ (0.51 vs 0.59). These comparisons emphasize the benefits of combining restricted aryl-N bond rotation with an EWG.
using ratiometric imaging, similar ON times allow a single camera integration time to be effective for acquiring data from both fluorophores. From these data, we determined that Yαle676b has an ON time of 4.5 ms at pH 7.4, which is close to the ~10 ms ON time reported for HMSIR, and in theory should allow even faster imaging. This short ON time, combined with the high quantum yield also makes the Yαle676b/HMSIR combination suitable for high-speed imaging, with camera frame rates as high as 400 frames per second (fps). With an OFF time of 3.8 s, we expect an ON fraction or duty cycle of 0.0012.

**Single-Color Live-Cell SMLM with Yαle676b**. We next tested whether Yαle676b would support single-color, live-cell SMLM imaging. U2-OS cells that were engineered to overexpress the endoplasmic reticulum (ER)-localized protein Halo-Sec61β were treated with 300 nM Yαle676b-CA (SI Scheme S8) for 30 min, washed, and immersed in a standard live-cell imaging solution using a custom-built SMLM instrument (see the SI discussion of methods). Figure 4a shows a representative super-resolution image (out of n = 16 images) acquired over 5 s. These images revealed multiple tubules in the cell periphery that were ~99 ± 15 nm (mean ± s.d.) wide, a value comparable to ER morphology metrics acquired using both STED and 4Pi-SMS. A time series illustrates changes in ER morphology that occur over the course of 10 s (Figure 4B). On average, we detected ~800 photons per blink, corresponding to a localization precision distribution with a peak at ~20 nm (Figure 4c,d).

**Ratiometric Two-Color Live-Cell SMLM with Yαle676b and HMSIR**. Next we sought to evaluate whether Yαle676b would support live-cell multicolor imaging in combination with HMSIR. U2-OS cells were transiently transfected with Halo-Sec61β (to reveal the ER) and SNAP-OMP25 (to reveal the outer mitochondrial membrane), treated with Yαle676b-CA and HMSIR-BG, and imaged using the identical SMLM setup. As predicted from the absorption and emission spectra of Yαle676b and HMSIR, both dyes could be excited with the same 642 nm laser and ratiometrically separated from two simultaneously acquired images detecting the emission wavelength ranges of 650–680 and 680–750 nm, respectively (SI Figure S5). Figure 4e shows a two-color super-resolution image, accumulated over 5 s, revealing the intertwined mitochondrial and ER networks of the cell. We detected comparable average photon numbers per frame for the two dyes (~500 and 590 photons for Yαle676b and HMSIR, respectively), especially given that the filters and excitation wavelength were optimized for HMSIR.

**CONCLUSIONS**

In summary, here we report a new spontaneously blinking Si-rhodamine, Yαle676b, that can be used alongside HMSIR to enable two-color ratiometric SMLM in living cells in physiological media. This new experiment was facilitated by three unique photophysical metrics associated with Yαle676b: (1) an exceptionally high quantum yield for a silicon rhodamine derivative (0.59); (2) an unusually long emission maximum (694 nm); and (3) a pKₐ value (5.9) that is nearly identical to that of HMSIR (6.0).

The unique photophysical metrics associated with Yαle676b result from the simultaneous introduction of both heterocyclic rings as well as electron-withdrawing dialky amino groups (DAGs) into the silicon rhodamine core. When either of these structural features is introduced in isolation, at least one of the
three critical photophysical metrics required for two-color SMLM becomes nonoptimal. Silicon rhodamine dyes with only heterocycle-containing dialkyl amino groups (such as HMSIR\textsubscript{red} and HMSIR\textsubscript{blue}) display long-wavelength emission (689–716 nm) but resist spirocyclization. As a result, their pK\textsubscript{cycl} values (6.4–9.0) are too high to ensure adequate distribution of single-molecule emitters (Figure 2). By contrast, silicon rhodamine dyes with only electron-withdrawing substituents, such as HMSIR\textsubscript{blue}, display a high quantum yield, but their spirocyclization equilibrium is too favorable, and their pK\textsubscript{cycl} values are too low (Figure 3). By combining these two substitution patterns in Yal\textsubscript{blue} and the competing effects on pK\textsubscript{cycl} are balanced, while the red shift from the heterocycle-containing DAG is maintained (Figure 3). Moreover, because both the rotational restriction from the heterocycle-containing DAGs and the electron-withdrawing capacity of the 2-fluoroethyl substituent inhibit twisted intramolecular charge transfer, the quantum yield increase from the latter is not only maintained, but enhanced (0.51 vs 0.59).

As expected, switching from a 2-fluoroethyl to a more electron-withdrawing 2,2-difluoroethyl substituent at the nitrogen in Cal\textsubscript{red} further increases the quantum yield, though at the expense of both pK\textsubscript{cycl} and emission wavelength (Figure 3). This pattern would likely continue with increasingly electron-withdrawing substituents. Despite these blue shifts, Cal\textsubscript{red} displays a comparable quantum yield to a previously reported and exceptionally bright Si-rhodamine fluorophore (compound 9 in ref 41), but with a >30 nm longer emission maximum.

Finally, we note that while the quantum yield increase relative to HMSIR observed with HMSIR\textsubscript{blue} is not as dramatic as that observed with Yal\textsubscript{blue} it is comparable to that observed from more commonly used azetidinyl substituents.\textsuperscript{16,35,36,45,46} Being that the former requires only one position at each aniline nitrogen to be substituted, whereas the latter requires two, use of the 2-fluoroethyl substituent may serve as an alternative method for increasing quantum yield of rhodamine derivatives, especially those with more complex DAGs. This approach and others described herein may serve as versatile methods for the preparation of even more greatly enhanced fluorescent labels.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssentsci.1c00670.

Description of all synthetic and imaging procedures and characterization of all fluorophores (PDF)

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**Notes**

The authors declare the following competing financial interest(s): J.B. discloses significant financial interest in Bruker Corp. and Hamamatsu Photonics.

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4. 4Pi-SMS for live cell imaging

The following section describes the design and implementation of a new 4Pi-SMS instrument for live-cell imaging. The new design specifically addresses several issues with the previous iteration including compatibility with a live-cell incubation chamber, a limited field of view, and an assortment of pain points identified through years of use.

My contribution to this work was identifying the design specifications, modifying the optical layout, updating the opto-mechanical design, improving the alignment protocol, constructing, and aligning the new system. The optical updates were a collaboration with George Sirinakis at the Gurdon Institute in Cambridge, UK. The mechanical updates were done in collaboration with Edward Allgeyer also at the Gurdon Institute in Cambridge, UK. The alignment updates were made with consultation from Yongdeng Zhang, now at Westlake University in Zhejiang, PR China.
4.1 4Pi-SMS

4Pi-SMS/iPALM is a SMLM technique that achieves unprecedented axial resolution in optical microscopy\textsuperscript{32,33}. By coherently interfering light from opposing sides of the sample with two objectives in a 4Pi geometry, the PSF intensity is modulated as a function of axial position (Fig. 4.1). At least two independent phase measurements must be taken because of the axial symmetry of the objective geometry. This means that at least three phase images must be produced because the phase images must sum to the original total intensity (Fig. 4.2).
Figure 4.1. Illustration of 4Pi-SMS concept. Axial position controls interference phase (a) which can be read out on a detector. In 4Pi-sms, this Interference phase modulates emitter brightness ($M_0$) on the camera (b). Intensity response reproduced from Aquino et al. 2011. Four different phase measurements are shown over the same range.
Figure 4.2. Examples of phase measurement schemes in iPALM (a) and 4Pi-SMS (b). iPALM scheme reproduced from Shtengel et al. 2008. 4Pi-SMS scheme reproduced from Aquino et al. 2011. In both cases, there is at least one resulting image that is degenerate. In iPALM, a custom 3-way beamsplitter is used to take
three images $\frac{2\pi}{3}$ from one another. In 4Pi-SMS, a clever use of polarization allows a combination of a Babinet-Soleil compensator, 50:50 beamsplitter, and a polarizing beamsplitter to take two conjugate pairs of phase measurements with an arbitrary phase shift between the two. Usually this is set to give four measurements $\frac{\pi}{2}$ from one another for more consistent localization precision across the axial range.

Recently, our group has implemented a variant of 4Pi-SMS that incorporates deformable mirrors (Fig. 4.3a). These adaptive optical elements shape the emitted light to control optical aberrations. In addition to correcting for optical aberrations induced by the instrument and sample, they are also used to add astigmatism for long-range 3-D localization (Fig. 4.3b). Salvaged fluorescence, a variant of ratiometric SMLM using the excitation/emission dichroic for ratiometric split, has also been implemented in 4Pi-SMS (Fig. 4.3c).
Figure 4.3. Recent developments in 4Pi-SMS. W4Pi-SMSN (a) incorporating deformable mirrors into the 4Pi-SMS geometry for aberration correction. Phase unwrapping (b) from astigmatic 3-D localization extends the axial range of 4Pi-SMS. Salvaged fluorescence (c) enables simultaneous multi-color imaging using far-red organic dyes. Mechanical layout with deformable mirrors from George Sirinakis. Phase unwrapping diagram reproduced from Huang et al. 2016. Salvaged fluorescence diagram reproduced from Zhang et al. 2020.

4.2 4Pi-SMS and live-cell imaging

While 4Pi-SMS as a technique is theoretically compatible with live-cell imaging, there are a few practical limitations. Because the sample must be sandwiched
between two high-NA objectives only 400 μm apart, physical space is quite limited. This is exacerbated in the initial design by the stage stack and optical layout. Another limitation of the system is its 18 μm field of view, which is not always large enough to image a whole eukaryotic cell, let alone an interacting pair.

To address these issues, I re-designed the system for live-cell imaging (Fig. 4.4-4.6). This redesign was based on a version by collaborators Edward Allgeyer and George Sirinakis at the Gurdon Institute in Cambridge, UK that was designed for use with 60X water immersion objectives for the imaging of *drosophila* egg chambers. In addition to the optical changes, I also incorporated several usability and alignment improvements to make the microscope more robust for daily operation as well as maintenance alignment.
Figure 4.4. 3-D view of new design. Front (a) and back (b) views of emission path mounted on the vertical breadboard. Samples are mounted inside the stage stack, left in (a) and right in (b). Excitation, objective lock, and alignment optics are all not shown but are on the optical table in the current implementation.
Figure 4.5. Revised optical layout. Emission-path optics occupy both the front (a) and back (b) of the vertical breadboard. 3-D view (c). Only the upper path is shown for clarity. Rays are colored by origin in the sample plane. Single-color intersections are image planes while multi-color intersections are pupil planes. Lenses are labeled with nominal focal lengths. QWP: quarter-wave plate, 50:50 BS: 50:50 non-polarizing beamsplitter cube, PBS: polarizing-beamsplitter cube, custom: Zemax-optimized custom lens described in section 4.4.
Figure 4.6. Revised mechanical layout. Front (a) and back (b) share the same vertical breadboard. The system is composed of three modules: the interference cavity combines the upper and lower emission paths to achieve single-molecule interference; the image-splitting module splits the two image pairs into four images; the camera relay module controls the final magnification and places all four images.
on the camera. The sample column refers to all elements along the objective axis passing through the sample.

**4.3 Sample access**

In the previous 4Pi-SMS design, the stage stack consists of 6 (sets of) stages: cavity Z, lower objective XY, upper objective Z, sample XY, sample course Z, and sample fine Z. Of these, the cavity Z stage pair is the most constricting because they are on either side of the stack and span the full travel range. To improve access to the sample area and facilitate live-cell chamber installation, the new design uses a single actuator for the cavity Z, supporting the stage stack only from below (Fig. 4.7).
Figure 4.7. Stage stack and sample column. In the new design (a), the cavity-scanning is performed by a single actuator supporting the stack from below and the number of optical elements in the sample column has been minimized. The old design (b) uses a pair of stages with supporting columns that flank the sample and has an additional lens and quarter-wave plate in each arm. Scale bar 50 mm.

In addition to reducing the stage stack, I also introduced additional space by minimizing the number of optical elements that enclose the sample. The previous layout had a lens and quarter-wave plate for each arm along the objective axis that have been moved to the main body such that each arm of the interferometer only has a single steering mirror in the sample column.
4.4 Optics

The major optical change to the interference cavity was the choice to under-fill the deformable mirrors. This has two benefits: improved performance with the 100X oil objectives used in the old system and compatibility with lower-magnification objectives. Because of the mechanism of the deformable mirrors, completely filling the pupil is suboptimal. Although the mirrors have a 12x12 array of actuators, there is a fixed boundary just beyond the outermost ones. Therefore, they only control the internal 11x11 array; under-filling allows improved control of the pupil. Under-filling also improves compatibility with lower-magnification objectives, which have larger pupils that would overfill the deformable mirrors with the old design, resulting in a clipped PSF.

After the cavity, the image splitting section between the two beamsplitter cubes was reduced to improve the optomechanics as described later. To accommodate this change without significantly increasing the optical path length and corresponding size of the instrument, the first lens after the cavity is not in the conventional 4f position. To compensate for this, I designed a custom lens with Zemax OpticStudio (Fig. 4.8). The lens was initially optimized using the global optimization toolbox to achieve the best performance possible at the first image plane outside the cavity. After all of the other optics were finalized, the custom lens
was again optimized to achieve the best performance in the final image plane (Fig. 4.9a). For comparison, the corner rays of a 40 μm square field are significantly clipped in the old design (Fig. 4.9b).
Figure 4.8. Zemax spot diagram analysis of intermediate image plane after the imaging cavity with (a) and without (b) custom meniscus lens. Blue crosses indicate traced rays normalized to airy discs (black circles; from Zemax). Rows correspond to rays coming from specified positions in the sample plane. Columns correspond to defocus in the image plane. All coordinates are μm in sample space. Diffraction-limited performance and field flatness are recovered through addition of a custom 5-mm thick BK-7 lens with Zemax-optimized curvatures.
Figure 4.9. Zemax spot diagram analysis of final image plane for new (a) and old (b) optical designs. Blue crosses indicate traced rays normalized to airy discs (black circles; from Zemax). Rows correspond to rays coming from specified positions in the sample plane. Columns correspond to defocus in the camera plane. All coordinates are μm in sample space. The spot diagrams in (b) for the rays from the corners of the 40 μm field of view were clipped entirely and can therefore not be displayed.
The last optical modification was a change to the pixel size. Previously, the system was optimized for a ~20 μm field of view that could be imaged at up to 800 Hz. To be able to monitor cell-to-cell interactions, we increased the field to 40 μm. Based on the readout speed limitations of modern sCMOS cameras, which can only read 256 lines of pixels at 800 Hz, this requires a pixel size of at least 156.25 nm. Because the distance between the last lens and the camera could not be reduced due to physical constraints, I increased the focal length of the previous lens to achieve this demagnification.

### 4.5 Optomechanics

When aligning a 4Pi-SMS system, the most challenging aspect is identifying exactly which component is out of alignment. This trouble-shooting requires significant prior understanding of the instrument design that a new builder will not have. This is complex enough that the written protocol recommends what is essentially a guess-and-check approach where the only recourse for a mis-aligned interference cavity is to repeat the process from the beginning. In our experience, this has led to huge variations in time-to-alignment, in one case taking more than a year.

To simplify the alignment troubleshooting, I constrained the system such that each step of the alignment has a single mirror (pair) that will achieve the desired effect.
This method ensures that a single iteration, done properly, converges to an aligned cavity. To this end, I removed the lenses and two of the mirror pairs between the beamsplitters. In the new design, the quadrant image-splitting is instead adjusted through movement of a single actuator that translates one mirror (Fig. 4.10). This also simplifies the alignment because the camera relay module no longer needs realignment after the quadrants are separated on the camera chip as per the published protocol.

![Figure 4.10. Image-splitting module.](image)

In the new design (a), a single translation mirror controls the splitting of the image pairs on the camera. Previously (b), this was done with a combination of three mirrors and subsequent re-alignment of the camera relay module.

The last set of optomechanical changes were made to reduce vibrations. Out of the six 4Pi-SMS systems using the old design, four had significant vibration issues. In
some cases these took several years to resolve, including multiple room-changes. To increase the system’s stiffness and corresponding resonant frequency, the vertical breadboard and all other structural elements were thickened by at least a factor of two. Additionally, the vertical cross-section was reduced to make the system less sensitive to the acoustic vibrations in the 5-100 Hz range thought to be the most problematic at Yale (Fig. 4.11). This was done by folding the beam to the back-side of the vertical breadboard for the final telescope.

**Figure 4.11.** Size comparison. New design overlaid on old shown in the same scale. Scale bar 50 mm.

### 4.6 Alignment
As in the published protocol, this system uses built-in pinhole positions rather than a beam profile system to significantly accelerate the alignment process. Additionally, the overview camera was decoupled from the alignment path so that the mirror in the flip mount could be replaced with a 50:50 beamsplitter plate. In the old design, the regular mirror blocks the alignment beam and forces users to align the lower arm using the beam from the top (in the reverse direction after removing the prism mirror), before being re-aligned in the forward direction with fluorescence from beads. Replacing this mirror allows both camera relay paths to be aligned simultaneously in the forward direction.

4.7 Protocol modifications

4Pi-SMS has been previously described in a protocol format\(^6\). Because of the design changes I have made, some adjustments should be made to the alignment protocol. They are as follows:

1. Because the alignment laser enters after the polarized beamsplitter, one translation mirror should be used as a reference while the other is used to match the two incident beams on the 50:50 non-polarized beamsplitter. This should be done at the start of cavity alignment.
2. Because the deformable mirrors are no longer the last mirrors before the non-polarized beamsplitter, they should be centered after the remainder of the interference cavity is aligned.

3. Because the stage stack uses a single base, it only needs to be aligned once. This should be done by matching the top objective thread to the beam from the upper arm of the interference cavity. The simplest way to do this is to make the upper alignment beam parallel to the objective axis and then translate the objective to match. Note that the two-plate base with orthogonal slots should aid in unidirectional translation of the stage stack.

4. Because the flip mirror introducing the alignment laser has been replaced with a 50:50 non-polarized beamsplitter, the lower camera relay arm can be propagated in the forward direction. This should be done before splitting the quadrant image pairs with the translation mirror. The subsequent forward alignment of the lower camera relay arm (step 138) should be skipped.

5. Both the objective lock and excitation should be assembled on the optical table. Note that the excitation path should be modified to double the size of the illumination to match the 40 μm square field. The default laser output powers should also be quadrupled in the control software to compensate for the increased area.
5. Other contributions

This thesis details my contributions to the field with regard to 4Pi-SMS and its use in live-cell imaging. My additional work can be found in publications pertaining to the implementation of a 4Pi-SMS system\textsuperscript{60} and optimizing labeling and imaging conditions in S. Pombe fission yeast\textsuperscript{61}.
In this thesis, I described my work towards live-cell 4Pi-SMS, where the major challenges have been controlling emitter density in living cells without cytotoxic effects and instrument-sample compatibility. First, I proposed a novel multi-color method for 4Pi-SMS using interference phase rather than dichroic elements. Next, I reported a new spontaneously-blinking fluorophore for live cell imaging without cytotoxic additives or near-UV irradiation. We also demonstrated its use in conjunction with HMSiR for two-color imaging in living cells. Finally, I described design changes to make 4Pi-SMS more robust and improve its live-cell imaging capabilities relative to previous implementations.

My work on identifying fluorophores by their interference phase opens the door to a new world of possibilities wherein fluorophores can be identified by features other than how their spectra split across dichroic elements. Such methods can be combined with conventional approaches to increase the number of fluorophores that can be imaged simultaneously. Additionally, my work suggests that the current 4Pi-SMS localization analysis and similar methods can be improved by factoring fluorophore spectra rather than treating them as monochromatic emitters with an empirically-derived characteristic wavelength.
On the front of live-cell SMLM, we have demonstrated 2-color imaging using organic dyes without deleterious chemical additives or near-UV irradiation. Now that we have opened the space of 2-color live-cell SMLM, the field will grow as others extend our work with their own biologies.

In addition to learning how to best label and image live samples for SMLM, we also gained insight into the critical features of an optimal live-cell organic dye. In particular, we learned the importance of being able to control emitter density during acquisition. Without a way to push fluorophores into an emissive state, the emitter density will simply decay exponentially due to photobleaching. To make the next breakthrough fluorophore for live-cell SMLM, we could release the constraints on pKa (that determine the on-fraction) and use the expanded chemical space to incorporate some degree of active control over the emissive state.

As it stands, I have laid the groundwork for live-cell 4Pi-SMS. I have built the instrument and also worked out how to use spontaneously-blinking fluorophores to label and image living cells. The next logical step is for biologists to bring their expertise and use my work to interrogate nano-scale 3-D interactions inside living cells.
While there are further instrumentation improvements that could improve performance, I believe the biggest gains in live-cell super-resolution will come from pushing temporal resolution through live-cell labeling strategies and extracting meaningful insights using the data already accessible with our current methods.

In particular, efforts should be directed towards:

**Cell-permeant fluorophores with tunable on-times and/or duty cycles**

Moving forward, the biggest challenge for live-cell imaging will be pushing temporal resolution. With my developments, our current instrumentation significantly outpaces the blinking capacity of our fluorophores. The currently available spontaneously-blinking fluorophores (HMSiR and Yale 676sb) have on-times of ~5 ms that cannot be changed and are consequently only suitable for imaging at ~200 Hz. In contrast, other SMLM modalities, like dSTORM, use fluorophores whose on-times are laser power-dependent. For example, the commonly used Alexa Fluor 647 has on-times that are inversely proportional to the excitation intensity, so that the on-time can be tuned as desired. However, the ~100 kW/cm² needed to reach on-times as short as 0.625 ms (for 1600 Hz imaging) are well beyond the threshold for phototoxicity. This approach also suffers from the reduction in dye performance at higher laser powers. While we have demonstrated that on-times and on-fractions can be tuned, the two are inevitably linked by the same chemical
equilibrium for spontaneously-blinking fluorophores. Therefore, the next generation of live-cell fluorophores should have some degree of independent optical/chemical control over both on-times and on-fractions. With such a (class of) fluorophore, the on-time can be shortened to match the camera acquisition rate for improved temporal resolution. The on-fraction could then be tuned orthogonally to meet the sparsity constraints of the fitting method. It is important to note that a suite of fluorophores could meet these criteria with one-dimensional tunability by spanning a range of values in the other. It is worth noting that our work suggests each of the fluorophores in this theoretical class would also have different excitation/emission spectra. This would complicate multi-color imaging because spectrally-separable fluorophores would then also have different blinking kinetics. However, most multi-color experiments have different temporal resolution requirements for each species of interest, with a “reference” channel that is significantly less mobile. These fluorophore developments would contribute to live-cell SMLM regardless of instrumentation.

**Novel live-cell emitter density control methods**

Alternative approaches to live-cell SMLM labeling have the potential to operate in a different blinking kinetic-space, possibly allowing access to regimes with enhanced temporal resolution. DNA-PAINT has analogously allowed microscopists to separate the blinking kinetics from fluorophore properties for fixed samples. LIVE-PAINT,
which uses reversibly-binding peptide tags, offers a similar potential in living cells\textsuperscript{63}. However, like DNA-PAINT, LIVE-PAINT relies on diffusion, limiting its capacity for high-speed imaging. Combining LIVE-PAINT with fluorogenic probes currently under development in our lab would remove the reliance on diffusion, which could significantly shorten on-times\textsuperscript{64}. As with fluorophore-specific modulation, new labeling schemes have the potential to enhance both 4Pi-SMS and standard SMLM.

**Multi-emitter fitting**

In conjunction with the developments mentioned above, multi-emitter fitting has the potential to significantly improve temporal resolution\textsuperscript{65}. Multi-emitter fitting would loosen the sparsity constraint, facilitating SMLM with higher emitter densities, albeit at the cost of spatial resolution. Increased emitter densities permit reconstructions with fewer camera frames, improving temporal resolution. Ideally these fitting methods would be generalized for standard SMLM as well as 4Pi-SMS.

**Computer vision**

The last free parameter we have to improve temporal resolution is the labeling density requirement. Computer vision has the potential to capture structures using lower information content than conventional image analysis methods\textsuperscript{66}. In segmenting a reconstructed image from live-cell time lapse recordings, it is possible
to utilize information from the entire sequence of raw frames. A first approximation of such a method would utilize temporal windowing for reconstruction such that each localization has an inclusion probability. Because time is not discrete, subsequent analyses using these modified reconstructions would outperform current methods that effectively use square windows\textsuperscript{67}. Even beyond the scope of the above proposals, these developments can be generally applied to all live-cell imaging. Indeed, there has already been significant work along these lines for pixel-based imaging modalities like MRI\textsuperscript{67}.

In the near future, progress in the field of super-resolution will come primarily from biologists who can design clever experiments that utilize the super-resolution tools we have already developed. Therefore, the biggest gains on the instrument side will come not from new technologies with minor improvements, but instead from usability improvements that enable a wider range of scientific inquiries.

For example, fully automated acquisition would significantly improve the throughput of highly informative data. Current instruments require expert users to set and adjust acquisition parameters like laser powers for optimal system performance. The converse of this is that the lion’s share of biologists are unable to acquire high-quality datasets. A convolutional neural network should be able to quickly learn the effects of these settings on the raw data. A second network could even be
trained to take in biological parameters, like temporal resolution, structure size, and expected labeling efficiency, to determine the optimal raw data conditions. The next step would be to use these two networks in conjunction with a cell atlas, enabling biologists to simply feed in their molecules of interest and corresponding labels to acquire optimal SMLM data. Adaptations of these networks should also be able to recommend fluorophores and labeling strategies based on biological questions.

Long-term, the biggest advances in live-cell imaging below the diffraction limit will come through enhanced labeling schemes. Because the performance of SMLM is fundamentally fluorophore/blink limiting, improvements in these two areas will shine through.
7. Bibliography

1. Leeuwenhoek A. *The select works of Antony van Leeuwenhoek containing his microscopical discoveries in many of the works of nature*. London: G Sidney; 1800.


8. Appendix

In this appendix, I provide the simulation scripts used in Chapter 2 as well as details about their use. I also include a section with notes as well as an old script used in my initial investigations into this space.

Theoretical monochromatic emitters

This block of MATLAB (2018A) code produces the plots in figure 2.1. It takes two arbitrarily-chosen wavelengths, then simulates the intensity, phase, and phase distance with a range of path-length differences that correspond to a full cycle.

```matlab
m = 1e6; % number of points
l = [3;5]; % wavelengths
dl = linspace(0,1,m)*prod(l); % path-length differences

%% intensity [au]
I1 = 1+sin(2*pi*dl/l(1));
I2 = 1+sin(2*pi*dl/l(2));

figure;
plot(dl,I1,'.',dl,I2,'.');

%% phase [pi]
phi1 = 2*permute(p(1,:,:),[1,3,2]);
phi2 = 2*permute(p(2,:,:),[1,3,2]);

figure;
plot(dl,phi1,'.',dl,phi2,'.');

ylim([0,2]);

%% phase distance [pi]
d = phi1-phi2;
d = min(abs([d;2-d;2+d]),[],1); % account for 2Pi wrapping

figure;
plot(dl,d,'.');

ylim([0,1]);
```

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Example fluorophores with spectra

The following block produces the plots in figure 2.2(a-c). It reads fluorophore emission spectra in the format provided by Semrock SearchLight (searchlight.semrock.com) to plot the expected intensity and phase distance as a function of path-length difference.

```matlab
%% Set parameters
m = 1e4;
dL = linspace(0,2.5e4,m);   % path-length difference [nm]
l = [580:630,660:710]';     % band pass limits [nm]
dye1 = 'AF647';
dye2 = 'CF568';

%% Read emission spectra
fn1 = fopen(strcat(dye1,' - EM.txt'));
Em1 = textscan(fn1, '%f %f', 'CommentStyle', '%');
Em1 = [Em1{1}, Em1{2}];
Em1 = Em1(ismember(Em1(:,1),l),2);
Em1 = Em1/sum(Em1);
fclose(fn1);

fn2 = fopen(strcat(dye2,' - EM.txt'));
Em2 = textscan(fn2, '%f %f', 'CommentStyle', '%');
Em2 = [Em2{1}, Em2{2}];
Em2 = Em2(ismember(Em2(:,1),l),2);
Em2 = Em2/sum(Em2);
fclose(fn2);

%% Simulate intensity
phi = 2*pi*dL./l; % phase (per wavelength, path-length difference)
I1 = [sum(Em1.*sin(phi),1);sum(Em1.*cos(phi),1)];
I2 = [sum(Em2.*sin(phi),1);sum(Em2.*cos(phi),1)];

%% Phase
phi1 = zeros(1,m);
phi2 = zeros(1,m);
parfor i = 1:m
    [x1,~] = im_phase(I1(i,:),1);
    [x2,~] = im_phase(I2(i,:),1);
    phi1(i) = x1(1)/(2*pi);
    phi2(i) = x2(1)/(2*pi);
end

%% Phase distance
```

dphi = phi1-phi2;
dphi  = min(abs(cat(3,dphi,1+dphi,1-dphi)),[],3); % 2pi wrapping

%% Comparison to characteristic wavelength
% emission peak used to match conventional fitting
phi1c = mod(dL./l(find(Em1==max(Em1),1)),1);
dphi1 = phi1-phi1c;
dphi1  = min(abs(cat(3,dphi1,1+dphi1,1-dphi1)),[],3); % 2pi wrapping

%% Save simulation results
save(sprintf('%s %s phase %dum %d.mat',dye1,dye2,max(dL),m));

%% Plot
figure; plot(dL,1+I1(:,1),'-',dL,1+I2(:,1),'-');
figure; plot(dL,2*dphi,'k.'
figure; plot(dL,2*dphi1,'.');

It uses the helper function im_phase, which is a simplified phase-fitting function based on the one used in conventional 4Pi-SMS or iPalm.

function [x,r]=im_phase(I,I0)
    options = optimoptions(@lsqnonlin,'Display','off');
    [x,r]=lsqnonlin(@fun,[pi,I0/2],[0,0],[2*pi,I0],options);
    function R=fun(x)
        R(1) = I(1) - x(2)*sin(x(1));
        R(2) = I(2) - x(2)*cos(x(1));
    end
end

**Glass comparison with emission spectrum**

Figure 2.2(d) is created using essentially the same code as above. Rather than comparing one fluorophore to another, it compares one fluorophore in vacuum to the same fluorophore in a selected glass.

%% Set parameters
m = 1e4;
dL = linspace(0,2.5e4,m);   % path-length difference [nm]
l = [660:710]';             % band pass limits [nm]
A = 'AF647';

fn1 = fopen(strcat(A, ' - EM.txt'));
Em1 = textscan(fn1, '%f %f', 'CommentStyle', 'f');
Em1 = [Em1{1}, Em1{2}];
Em1 = Em1(ismember(Em1(:,1),l),2);
Em1 = Eml/sum(Eml);
fclose(fn1);

%% Refractive indices by wavelength for selected glasses

12 = (1/1e3).^2;

SF11
n = sqrt(1+1.73759695*l2./(l2-0.013188707) + ...
   0.313747346*l2./(l2-0.0623068142) + ...
   1.89878101*12./(l2-155.23629));

BK7
n = sqrt(1+1.03961212*l2./(l2-0.00600069867) + ...
   0.231792344*l2./(l2-0.0200179144) + ...
   1.01046945*12./(l2-103.560653));

%% Simulate intensity

phi = 2*pi*dL./l; % in vacuum, n=1
phin = 2*pi*(dL/mean(n)).*n./l;
I1  = [sum(Eml.*sin(phi),1);sum(Eml.*cos(phi),1)]';
I2  = [sum(Eml.*sin(phin),1);sum(Eml.*cos(phin),1)]';

%% Phase

phil = zeros(1,m);
phi2 = zeros(1,m);
parfor i = 1:m
    [x1,~] = im_phase(I1(i,:),1);
    [x2,~] = im_phase(I2(i,:),1);
    phil(i) = x1(1)/(2*pi);
    phi2(i) = xB(1)/(2*pi);
end

%% Phase distance

dphi = phi2 - phil;
dphi = min(abs(vertcat(dphi,1+dphi,1-dphi)));

save(sprintf('%s dispersion %dum %d.mat',A,max(dL),m));

%% Plot

figure; plot(dL,2*dphi,'.');

Experimental validation

Should the need arise, this method can be validated experimentally by acquiring DNA-origami data with two different fluorophores simultaneously. For illustration, it
would be best if these two fluorophores are on distinct structures that are both flat. A good example of this would be DNA Origami frame and rectangular array structures. To this end, the new 4Pi-SMS system has HiLo/’dirty TIRF’ excitation available.

When setting the system for acquisition, move the cavity stages to the intensity maximum offset by half the expected path-length difference to the first phase distance maximum. That is, if interference contrast is maximized at \( z \) (path-length difference \( = 0 \)) while the first phase distance maximum occurs at \( \Delta L \), the cavity stages should be set to \( z \pm \Delta L/2 \). In this position, the sample should be at the z-position where the phase distance is maximized.

When analyzing these data, the normal 4Pi-SMS analysis should appear to show the structures in two different z-positions. This is because the fine z-position is determined linearly as a function of phase. This should be sufficient to demonstrate that the method is working. For actual use, the fit needs to be modified to simultaneously fit phase and z-position, as in Jonas Ries’ code (currently in development).
Other notes

Below are notes I took in my earlier investigations of this space. I have also included the script that I used in the past to investigate the possible methods for applying this and their potential tradeoffs. Note that the resolution compromise expectations are approximations based on using the current method without separate phase and z-position fitting.

Notes on filter-less dye identification with 4Pi-SMS nanoscopy

- 4Pi-SMS provides four images—for each point, there should be four intensities (per pixel in the psf). This means that there are four equations for three unknowns given a single color. Unfortunately, one of these is degenerate since all four can be generated from only three parameters.
- To solve the degeneracy, a constraint can be added that bounds the z-localization with the astigmatism. This is essentially how it is currently used, but has uncertainty based on the degree of astigmatism applied.
- Once you have confined z (to a 100-nm range) with the astigmatism, the absolute phase (between analogous components from the two dyes) matters and can create separation.
- This separation can be created using the dispersion in the two glasses. First the quartz wedge distance is set to provide a π/2 shift between S and P polarizations, ideally for all wavelengths within the bandpass (chosen for the mean of the peak emissions in the simulation). After that, the
BK7 wedge is set to achieve the dispersion necessary to distinguish the two spectra and the air path length is changed to center the coherence of the emissions.

- Using the path lengths and the emission spectra of the dyes (modified by the barrier filter), you can simulate the emissions of a specific fluorophore at a given z position. In the simulation, Poisson shot noise is added followed by Gaussian read noise.

- These simulated data can be fit to each of the different emission spectra and assigned to the fluorophore with the lowest error. This tells you how well the fluorophores are separated. Transverse resolution is ignored (simulating the psf as a single pixel), but the width of the distribution of z-localizations was used as a measure of axial resolution.

Conclusions

- As you increase the phase angle separation between the (two) fluorophores, crosstalk diminishes. Given the spectra of AF647 and CF680 (which can be excited by the same laser) and a bandpass centered at 700 nm (width 70nm), perfect color assignment over the range [-600,600] occurs with a minimum separation of 2.36 (about \(3\pi\)). As the previous statement would suggest, this is a function of the emission spectra (modified by the bandpass) along with the z range that we wish to acquire of the sample.
Increasing dispersion unfortunately also reduces coherence—this attenuates the signal, reducing the SNR and worsening axial resolution. At complete separation, this amounts to a 2-2.5x change in resolution. This is also color-dependent since it depends on the coherence. In this example, AF647 has a longer coherence length since its spectrum is sharper due to the bandpass. This makes the resolution better for AF647 than CF680 in the setup used.

Axial resolution also changes with depth. As you progress in the direction of increasing path length, this effectively increases the phase separation between wavelengths, meaning that for overall separations near the threshold, you can see a range across z: at one end, you have worse resolution but better identification while at the other you get better resolution (still worse than normal) with poor identification.

Outlook

Without changing the 4Pi setup, colors can only really be separated by dispersion, which worsens resolution. This dictates that we must minimize the phase separation while still maintaining a minimal acceptable fluorophore identification rate.

This minimal phase separation depends on three things. Coherence (spectrum width, spectral distance between fluorophores, band pass cutoff), z-range, and the astigmatism.
Re: spectrum width – the narrower the spectra, the longer the coherence length and consequently the better the resolution at increasing phase shifts. Unfortunately, this is hard to control and there are a finite set of dyes (it would work extraordinarily well for something like quantum dots, but those are not suitable).

Re: spectral distance – if the two fluorophores are well-separated, then less dispersion is required to achieve the same separation. This results in a reduced phase shift, improving the resolution for a given spectra. Unfortunately, if the two dyes are not close spectrally, they cannot both be excited by the same laser; this would require setup modification (and at least one more laser).

Re: band pass width – If the band pass is narrowed, it would cut off more of the tails, increasing coherence length. This would improve resolution, but may harm color separation as non-overlapping regions are attenuated. Additionally, this would significantly harm transverse resolution because it would throw away photons, which are already precious.

Re: z-range – if we decrease the imaging range, we would not need to maintain the color separation for as wide a range of depths. This allows us to reduce the phase shift, improving resolution. Sadly, this requires removing one of the strengths of the 4Pi.
Re: astigmatism – this is used to place bounds on the z-range; increasing astigmatism (slope) will narrow the bounds of the fitting. This will mean that less phase separation is required to differentiate the fluorophores. Unfortunately, increasing astigmatism appears to worsen resolution at exactly the same rate that it would be improved.

Potential solutions: Other PSF engineering (similar to the astigmatism, potentially higher-order), improving coherence over the z-range.

Script as mentioned above:

```matlab
%% Set and solve for parameters
clear
clc

m = 10;                  % number of runs
l = [575:630,670:730]';    % band pass limits [nm]
z = (-1000:1000)';         % z range [nm]
A = 'CF568';
B = 'CF660C';

% Simulation Parameters
I0  = 2000;        % total signal [photons]
b   = 0;           % mean background [photons]
sig = 6.5;         % stdev gaussian noise [photons]
a   = 50;          % bounding from astigmatism [nm]
n   = 1.33;        % sample refractive index
dphi0 = pi;
dphiX = pi/4;       % phase between P/S, typically pi/4

fEmA = fopen(strcat(A,' - EM.txt'));
EmA = textscan(fEmA, '%f %f', 'CommentStyle', '%');
EmA = [EmA{1}, EmA{2}];
lA = EmA(EmA(:,2)==1,1);
EmA = EmA(ismember(EmA(:,1),l),2);
fclose(fEmA);

fEmB = fopen(strcat(B,' - EM.txt'));
EmB = textscan(fEmB, '%f %f', 'CommentStyle', '%');
EmB = [EmB{1}, EmB{2}];
lB = EmB(EmB(:,2)==1,1);
EmB = EmB(ismember(EmB(:,1),l),2);
fclose(fEmB);
```

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\[ l_X = \frac{1A + 1B}{2}; \quad \% \text{wavelength where } d\Phi = \pi/4 \text{ from quartz} \]

\[ l_2 = (1/1000)^2; \]
\[ l_{A2} = (1A/1000)^2; \]
\[ l_{B2} = (1B/1000)^2; \]
\[ l_{X2} = (1X/1000)^2; \]
\[ k = 2\pi n./l; \quad \% \text{phase-wrapping distance [nm]} \]

\[ n_{BK7a} = \sqrt{1 + 1.03961212 \times 1A2 ./ (1A2 - 0.00600069867) + 0.231792344 \times 1A2 ./ (1A2 - 0.0200179144) + 1.01046945 \times 1A2 ./ (1A2 - 103.560653)}; \]
\[ n_{Qea} = \sqrt{1.28851804 + 1.09509924 \times 1A2 ./ (1A2 - 0.0102101864) + 1.15662475 \times 1A2 ./ (1A2 - 100)}; \]
\[ n_{BK7b} = \sqrt{1 + 1.03961212 \times 1B2 ./ (1B2 - 0.00600069867) + 0.231792344 \times 1B2 ./ (1B2 - 0.0200179144) + 1.01046945 \times 1B2 ./ (1B2 - 103.560653)}; \]
\[ n_{Qeb} = \sqrt{1.28851804 + 1.09509924 \times 1B2 ./ (1B2 - 0.0102101864) + 1.15662475 \times 1B2 ./ (1B2 - 100)}; \]

\[ n_{BK7x} = \sqrt{1 + 1.03961212 \times 1X2 ./ (1X2 - 0.00600069867) + 0.231792344 \times 1X2 ./ (1X2 - 0.0200179144) + 1.01046945 \times 1X2 ./ (1X2 - 103.560653)}; \]
\[ n_{Qox} = \sqrt{1.28604141 + 1.107044083 \times 1X2 ./ (1X2 - 0.0102101864) + 1.10202242 \times 1X2 ./ (1X2 - 100)}; \]
\[ n_{Qex} = \sqrt{1.28851804 + 1.09509924 \times 1X2 ./ (1X2 - 0.0102101864) + 1.15662475 \times 1X2 ./ (1X2 - 100)}; \]
\[ Dn_{Qx} = n_{Qox} - n_{Qex}; \]
\[ n_{BK7} = \sqrt{1 + 1.03961212 \times 1L2 ./ (1L2 - 0.00600069867) + 0.231792344 \times 1L2 ./ (1L2 - 0.0200179144) + 1.01046945 \times 1L2 ./ (1L2 - 103.560653)}; \]
\[ n_{Qo} = \sqrt{1.28604141 + 1.07044083 \times 1L2 ./ (1L2 - 0.0100585997) + 1.10202242 \times 1L2 ./ (1L2 - 100)}; \]
\[ n_{Qe} = \sqrt{1.28851804 + 1.09509924 \times 1L2 ./ (1L2 - 0.0102101864) + 1.15662475 \times 1L2 ./ (1L2 - 100)}; \]
\[ Dn_{Q} = n_{Qo} - n_{Qe}; \]

% Path difference (upper - lower) [nm]
\[ dL_Q = d\phi_X / \pi \times 1X ./ Dn_{Qx}; \]
\[ dL_{Air} = (d\phi_0 / (2\pi) + (n_{Qex} \times n_{BK7b} \times dL_Q) / (n_{BK7x} \times 1B) - (n_{Qeb} \times dL_Q) / 1B - (n_{Qex} \times n_{BK7a} \times dL_Q) / (n_{BK7x} \times 1A) + (n_{Qea} \times dL_Q) / 1A) / ...\]
\[ \quad (-n_{BK7b} / (n_{BK7x} \times 1B) + 1/1B + n_{BK7a} / (n_{BK7x} \times 1A) - 1/1A); \]
\[ dL_{BK7} = -(dL_{Air} + n_{Qex} \times dL_Q) / n_{BK7x}; \]
\[ dL = dL_{Air} + dL_Q \times n_{Qe} + dL_{BK7} \times n_{BK7}; \]
\[ \phi_0 = 2\pi \times dL ./ 1; \]
\[ d\phi = 2\pi \times dL_Q \times Dn_{Q} ./ 1; \]

% Traditional method
% \phi_0 = 0 \times \phi_0;
% \frac{d\phi}{\pi} = \frac{\pi}{2} \times \text{ones(size(d\phi,1),size(d\phi,2))};

% Monte Carlo
\[ o = \text{size(z,1)}; \]
\[ IA = \text{zeros(o,4,m)}; \]
\[ IB = \text{zeros(o,4,m)}; \]
for i = 1:o
    IA(i,1,:) = I0/2*dot(EmA,(cos(2*(z(i))*k+phi0)+1))/sum(EmA);
    IA(i,2,:) = I0/2*dot(EmA,(cos(2*(z(i))*k+phi0+dphi)+1))/sum(EmA);
    IA(i,3,:) = I0/2*dot(EmA,(cos(2*(z(i))*k+phi0+pi)+1))/sum(EmA);
    IA(i,4,:) = I0/2*dot(EmA,(cos(2*(z(i))*k+phi0+dphi+pi)+1))/sum(EmA);
    IB(i,1,:) = I0/2*dot(EmB,(cos(2*(z(i))*k+phi0)+1))/sum(EmB);
    IB(i,2,:) = I0/2*dot(EmB,(cos(2*(z(i))*k+phi0+dphi)+1))/sum(EmB);
    IB(i,3,:) = I0/2*dot(EmB,(cos(2*(z(i))*k+phi0+pi)+1))/sum(EmB);
    IB(i,4,:) = I0/2*dot(EmB,(cos(2*(z(i))*k+phi0+dphi+pi)+1))/sum(EmB);
end

% add shot noise
IA = IA + poissrnd(sqrt(IA));
IB = IB + poissrnd(sqrt(IB));

% add read noise
IA = IA + normrnd(b*ones(o,4,m),sig*ones(o,4,m));
IB = IB + normrnd(b*ones(o,4,m),sig*ones(o,4,m));

%% Fit for Color-attribution and resolution
rAA = zeros(o,m);
rBB = zeros(o,m);
rAB = zeros(o,m);
rBA = zeros(o,m);
zA  = zeros(o,m);
zB  = zeros(o,m);

parfor i = 1:o
    if ~mod(i,50)
        fprintf('%d
',i);
    end
    for j = 1:m
        [xA, rAA(i,j)] = phase_4Pi_spectrum(IA(i,:,j),phi0,dphi,k,z(i),I0,a,b,EmA);
        [xB, rBB(i,j)] = phase_4Pi_spectrum(IB(i,:,j),phi0,dphi,k,z(i),I0,a,b,EmB);
        [~, rAB(i,j)]  = phase_4Pi_spectrum(IA(i,:,j),phi0,dphi,k,z(i),I0,a,b,EmB);
        [~, rBA(i,j)]  = phase_4Pi_spectrum(IB(i,:,j),phi0,dphi,k,z(i),I0,a,b,EmA);
        zA(i,j) = xA(2);
        zB(i,j) = xB(2);
    end
end

zAstd = std(zA,0,2);
zBstd = std(zB,0,2);
fAB = mean(rAA>rAB,2);
fBA = mean(rBB>rBA,2);

save(sprintf('%s %s m=%d dphi0=%d single.mat',A,B,m,dphi0));

%% Plot!
figure;
plot(z,fAB,'.',z,fBA,'.');
title(strcat({'Fraction misattributed with $\Delta\phi_0 = '},num2str(dphi0))

figure; subplot(2,1,1)
plot(z,mean(rAA,2),z,mean(rAB,2),'.')
title(sprintf('%s Residues',A)); legend(A,B)
subplot(2,1,2)
plot(z,mean(rBA,2),'.',z,mean(rBB,2))
title(sprintf('%s Residues',B)); legend(A,B)

figure;
plot(z,20/.25*zAstd,'.', z,20/.25*zBstd,'.')
title(strcat({'Resolution v. Depth for $\Delta\phi_0 = '},num2str(dphi0),{', n = '},num2str(m)))
xlabel('Z (nm)');
ylabel('Z Resolution (nm)');
legend(A,B);