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Project Information

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Institution: University of Arkansas

Classification: Junior

Grade Point Average: 4.0

Area of Study: Chemistry, Physics

Title of Project: Single Molecule Spectroscopy and Spectral-Resolved Super-Resolution Imaging by Spectral Phasor Approach

Abstract and Summary

Single-molecule localization microscopy (SMLM) has become a strong technique in the toolbox of chemists, biologists, physicists, and engineers in recent years for its unique ability to resolve characteristic features quickly and accurately in complex environments at the nanoscopic level. Multicolor super-resolution imaging has seen the greatest advancement among SMLM techniques, drastically improving the differentiation ability of nanostructures beyond the diffraction limit and increasing the precision with which previously unresolvable structures are studied. However, traditional multicolor SMLM methodologies present low spatial resolution and suffer low photon efficiency.

Here, I propose a solution to these drawbacks by developing an ultrahigh-throughput SMLM methodology that allows for fast and accurate multicolor imaging of complex environments at the nanoscopic level using a multichannel system paired with phasor analysis.

To create this system, *I have outlined five main aims of the project.*

1. Integrate our previously developed three-channel imaging box into a TIRF microscope.
2. Replace key components of the microscope and imaging box with those more suitable with single-molecule spectroscopy.
3. Calibrate our setup by employing many different fluorophores with different emission peaks and profiles.
4. Different fluorophores will be tested on each subcellular components of interest (mitochondria, microtubules, clathrin-coated pits, etc.) to find the best for each structure.
5. Fixed cells will be tagged at the components of interest with the best fluorescent dyes. Super resolution imaging data will be taken and analyzed to validate the methodology.

This method has the potential to overcome traditional SMLM issues by negating the need to separate signals used for spectral and localization analysis, permitting ultrahigh-throughput with high spatial and spectral resolution and true color.

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Single Molecule Spectroscopy and Spectral-Resolved Super-Resolution Imaging by Spectral Phasor Approach

Background and Significance.

Super-resolution microscopy has gained widespread attention across multiple research areas[1-3] due to its ability to produce high spatial-resolution images that were formerly only achieved through electron microscopy. Single-molecule localization microscopy (SMLM)[4] has been at the forefront of super-resolution methodologies in the last decade. The level of spatial resolution provided by SMLM reconstruction techniques reveal previously unresolvable kinetic, dynamic, and structural information of sophisticated biological[1, 4], chemical[2], and material samples[12] at nanometer scale and single molecule level. For example, Figure 1 shows the comparison between a traditional epifluorescence image (spatial resolution 200-350 nm) and the analogous super-resolved fluorescence image (spatial resolution 10-50 nm). As shown in the figure, the latter method gives a much clearer picture of the subcellular structures.

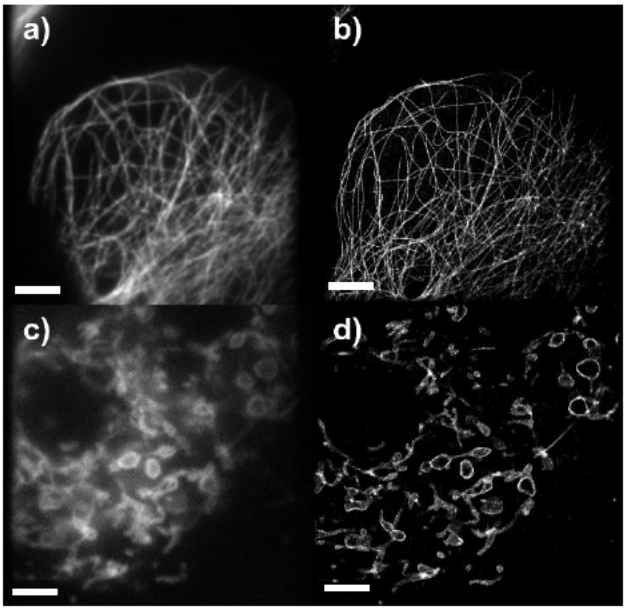

Figure 1: a) epifluorescence image of β-tubulin. b) SMLM image of β-tubulin. c) epifluorescence image of mitochondria. d) SMLM image of mitochondria. Scale bar 5 μm. [redacted] 2023).

Combinatorial spectroscopic methodologies have been developed to use in combination with SMLM and create novel spectro-microscopic systems capable of simultaneously acquiring high-resolution images and spectral features at the single-molecule level[8]. Commonly, this is accomplished by employing spectral dispersion elements such as gratings or prisms that provide full spectrum analysis[8]. Using these, a spectrally resolved super-resolution image can be obtained. In these methods, diffraction gratings or prisms separate the acquired signal spatially and spectrally, guiding these components separately to the

detector to localize and spectrally resolve emission events of single fluorophores.[8] These methodologies allows the full emission spectrum to be obtained from single molecules. However, these techniques suffer from low throughput and low photon efficiency. Low throughput is due to spatially overlapping spectra between fluorophores in close spatial proximity. The achievable spatial resolution in SMLM depends on the collected photons from single molecules with a relationship of $\sigma \sim N^{-1/2}$.[5] For spectral dispersion-based methods, most of the collected photons are spatially dispersed in the spectral channel to improve the accuracy of wavelength determination. Only a small portion of photons are kept for localizing the exact positions of molecules, thus limiting the achievable spatial resolution.

Proposal Goal. I propose a solution to overcome the issue of poor photon efficiency and low throughput presented in the previously mentioned spectro-microscopic techniques. I will incorporate phasor analysis for multicolor super resolution imaging. The phasor approach is a data analysis and modification technique that creates vectorial representations of sinusoidal waves. These vectors are linear combinations of the real (G-vector) and imaginary (S-vector) parts of a wave which can be plotted on a phasor plot[1,4,10]. The angle between the vector and the x-axis of the plot corresponds to the peak wavelength, the magnitude of the vector determines the width of the spectra[1,4]. Conventionally, the G and S components of the vector are obtained through a Fourier transform using cosine and sine functions. However, such analysis can also be done by directly passing fluorophore emission through optical filters whose transmission profiles emulate sine and cosine functions. By this method, signal is split into three channels (reference, sine, and cosine) and is transformed before collection by the detector. Then, only a simple calculation is needed to find G and S (Eq. 1,2[6]).

$$G = 2\frac{\frac{I_{cos}}{I_0} - T_{cos/min}}{T_{cos/max} - T_{cos/min}} - 1 \qquad 1)$$

[redacted]

$$S = 2\frac{\frac{I_{sin}}{I_0} - T_{sin/min}}{T_{sin/max} - T_{sin/min}} - 1 \qquad 2)$$

Where I_n are photon intensities in their respective channels, T_n are the associated transmission maxima and minima in each filter.

With plotted vectors, the peak wavelength and distribution width of the emitted signal can be obtained per fluorophore. This method solves the issues of low throughput and photon efficiency by reducing the need for a dispersion element. It eliminates the need for single molecules to be very far separated which is required for the dispersion method. Therefore, spectrally resolved SMLM with high molecular density can be achieved. Meanwhile, photons from all channels can be combined to improve the localization precision thus allowing better usage of photons for higher spatial resolution.

Experimental plan. I have previously developed a spectral phasor method for single particle spectroscopy analysis of metal nanoparticles[6] (Figure 2). In this proposal, I plan to modify and optimize this method for single molecule spectroscopy analysis and further establish an ultrahigh-throughput and spectrally resolved super-resolution microscopy imaging methodology.

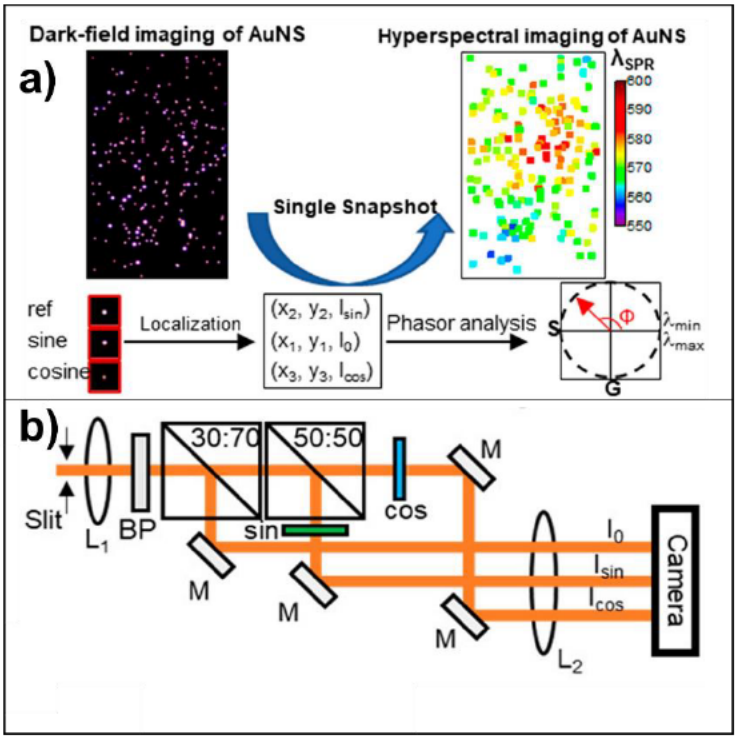

Figure 2: a) Overview of method. Intensity from 2 modified and 1 reference channel are plotted on phasor plot, then peak wavelength and width can be extracted from each frame. b) Imaging setup of previous spectral phasor work on nanomaterials using sin/cos filters. ([redacted]

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My detailed plan is as follows:

1. I will begin by integrating the previously developed three-channel system designed for single particle spectroscopy to a TIRF microscope capable of super resolution imaging.
2. Then, the optical components will be replaced with those that are more suitable for the wavelength ranges used in single-molecule fluorescence imaging.
3. Several fluorophore types of different peak wavelengths will be imaged on coverslips to calibrate the system's phase angle relation to wavelength and spectral resolution.
4. Subcellular structures will be tagged with a variety of fluorescent dyes to determine which is the best for each structure. At this point, analysis of spatial resolution will also be determined.
5. Fixed cells will be tagged with all selected fluorescent dyes and super resolution imaging data will be taken.

Personal Background and Involvement. While pursuing my degrees in chemistry and physics, under the direction of Dr. [redacted] I have taken part in multiple projects relating to optical microscopy, methodology development, single particle tracking, super resolution, and more. I am first author on another work that is based on the phasor approach to imaging spectroscopy [redacted] and am currently authored on two additional submitted manuscripts. I have presented 6 posters at conferences such as ACS National, GRC, and more, and I have given talks in our Analytical Chemistry Division Seminar as well as recently being invited to speak at Arkansas INBRE 2023 regional meeting.

In this project, I will be leading the development of the methodology. This includes sourcing components, testing/calibration, experimentation, data analysis, coding, and more. Application studies with cell imaging will be carried out by myself and other members of the group.

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Plan and Project Timeline

Month	Goal(s)
January	• Modify 3 channel imaging box for super resolution imaging.
February	• Modify microscope with imaging box – test.
March	• Fluorophore testing to find best dyes for our case.
April	• Single color fixed super resolution imaging (validation).
August	• Multi-color fixed super resolution imaging – testing conditions for cells, media, and tagging.
September	• Experimental multi-color SMLM imaging of fixed A549 cells with selected fluorescent dyes.
October	• Continue experiments, write codes for data analysis, and analyze data.
November	• Finish data analysis, begin writing manuscript and submitting abstracts for conference talks.

Conclusion and Future Work

Fluorescence microscopy is invaluable to researchers and professionals alike. Current super resolution methodologies vastly improve upon traditional epi-fluorescent imaging techniques, but there is still considerable room for improvement. Here, I propose a new stochastic super resolution methodology capable of solving key issues present in current multicolor SMLM techniques. Without this work, ample improvements will be left behind that could improve both the temporal and spatial resolution of current fluorescent technique. In the future, I plan to use this instrument to carry out other projects in super resolution, super resolution single particle tracking, and a variety of other studies important to the biological, chemical, and materials science fields.

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