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Two-Color Fixed Cell Imaging Using Engineered Point Spread Functions – XPSF Family

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Superresolution fluorescence microscopy plays a prime role in understanding the processes at the cellular level as a probe that allows visualizing multiple nanoscale organelles simultaneously at a high resolution. Engineering the point spread functions (PSFs) that a fluorescence emitter imprints on a camera so that the PSF itself consists of spatial and spectral information is of great interest for this would allow imaging without emission filters and multiple cameras in the optical path of a microscope [1]. Thus, the limited photon budget of a fluorescently tagged cell sample can efficiently be utilized at a lower cost. In addition, an optimized family of PSFs would allow using fluorescent labels that excite with a single laser and have closely overlapping emission spectra that usually cannot be separated using emission filters [2].

We have previously introduced a family of PSFs that we named XPSFs that simultaneously distinguish between spectra and localize in space [3]. The XPSFs are formed with a four-quadrant glass phase plate with diagonal quadrants having a similar thickness, placed at the Fourier Plane of the microscope. Here, we demonstrate two-color fixed cell imaging results using this simple, comparatively less expensive phase plate. We imaged TOMM20 tagged in CF568 dye (excited by a 561 nm laser line), and microtubules tagged in Alexa Fluor 647 (excited by a 640 nm laser line) in fixed U2OS cells using Vutara superresolution microscope after incorporating the phase plate accordingly. We acquired the data PSFs following the same procedure as usual dSTORM imaging, but the lasers were simultaneously exciting the sample. Simultaneous excitation has some more challenges compared to sequential: for instance, the backgrounds from the sample are prominent, the lasers need to be controlled so that the PSFs are sufficiently sparse at a camera frame while maintaining an adequate number of photons per PSF, and picking the regions of interest (ROIs) that enclose the PSFs is tricky due to different relative intensities and PSF sizes.

Once we captured the data frames, we used a homebuilt Matlab algorithm to distinguish each ROI in x,y,z spatial coordinates, and a representative wavelength. We took a simple approach by modeling the expected PSFs using the scalar Gibson-Lani PSF model [4] and Maximum Likelihood Estimation to match the data PSFs with the model [5]. We calibrated the model for Zernike aberrations via data PSFs obtained from Thermofisher Tetraspeck calibration beads. Then we rendered the final image in Figure 1 using the Vutara SRX software.

We also conducted a controlled experiment to obtain an idea about the spectral discernibility of the XPSFs by identifying whether a probe is CF568 or Alexa Fluor 647 (AF) by obtaining data *with* emission filters at sequential excitation and executing the same localization procedure. We report 97%(AF647) and 88%(CF568) correct localizations using only XPSFs at an average background level of 125 photons per camera pixel and 3000 photons per PSF at a [-400,400] nm depth. The plot in Figure 2 shows the results of a Monte-Carlo simulation using noisy PSFs that demonstrates the expected

performance of the XPSFs at different background levels at 3000 photons per pixel. We attribute the differences between the simulation and the actual to the accuracy of model calibration, the level of Zernike aberrations that reduce the PSF shapes' differences, and errors in estimating the number of photons per PSF. We also report a 25 nm x,y resolution by considering the full width at half the maximum value of the distribution of localizations for microtubule cross-sections and a limited 300 nm z resolution.

In conclusion, the XPSFs can localize emitters spatially and spectrally with limitations. We are trying to obtain three-color images using the XPSFs and optimize the PSFs for better imaging capability [6].

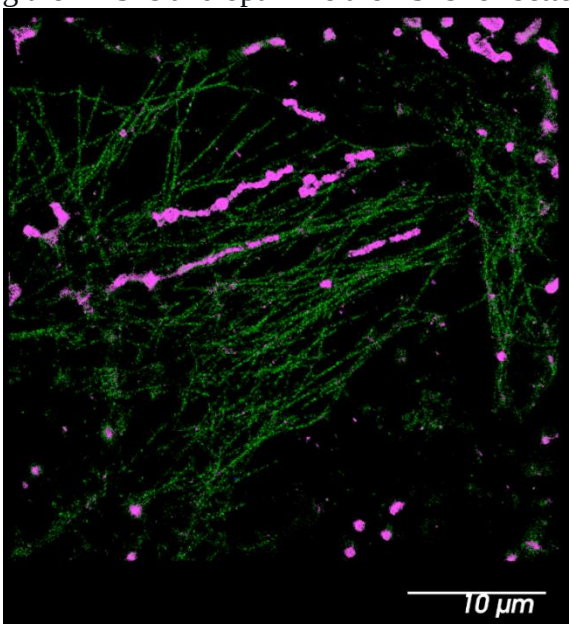

Figure 1. Two color fixed cell image using XPSFs. Microtubules (AF647 tag) are in green and mitochondria (TOMM20) (CF568 tag) are in pink. The 561 and 647 nm laser lines excited the sample simultaneously and the image was rendered using 5000 camera frames captured at 40 ms rate.

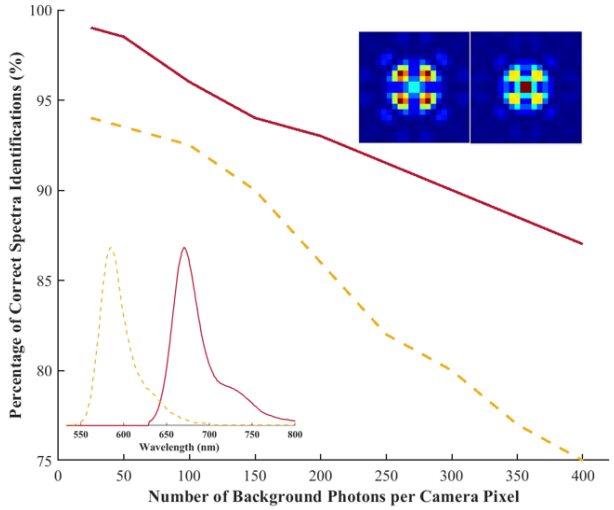

Figure 2. Monte-Carlo simulation results demonstrating the expected performance of the XPSFs when the background changes. The dashed orange line corresponds to CF568 and the magenta continuous line

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corresponds to AF647. The two spectra are at the left bottom and the model PSFs for AF647 and CF568 at zero x,y,z are at the top right.

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