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A Proposed Method for Optimizing the Spectral Discernibility of Engineered Point-spread Functions for Localization Microscopy

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Localization microscopy has become an established tool for investigating protein distributions within cells at resolutions around 20 *nm* [1-3]. An outstanding challenge within the field stems from spectral width of the fluorescent markers used to tag proteins of interest – generally about 50 *nm* FWHM. This width decreases the number of protein species it is possible to tag and simultaneously observe without incurring unacceptable cross-talk between spectral channels. Given how dynamic and interconnected all of the functions of a live cell are, this is a significant limitation for future live-cell imaging applications. Additionally, markers with spectral centeres in the range of 650-750 *nm* produce better signal to noise ratios than do bluer dyes due to the low interaction between red light and the cellular environment. Thus, multi-color experiments with red dyes would be ideal in this sense but their spectral widths and the resulting cross-talk currently make such experiments impossible.

Recently, we and others [4-7] have begun using point-spread function (PSF) engineering, rather than traditional filters, to separate spectral channels. By introducing a phase mask into the Fourier plane (FP) of a microscope (Fig. 1.c), the PSF can be made very sensitive to emission spectra. In this way we hope to image many fluorescent tags simultaneously with negligible cross-talk. To optimize such a PSF, we introduce a figure of merit (FOM) called *pixel confusion*, which characterizes the probability that a given pixel will have the same photon count for two different emission spectra.

More specifically, suppose two PSFs, X_1 and X_2 , are centered on the same region of a camera. If X_1, X_2 have different spectra then their PSFs will differ based on the design of the phase plate. If the photon count, x_i , of the i^{th} pixel between the measurements of X_1 and X_2 is the same, then we say that the i^{th} pixel is *confused*. Assuming Poissonian detection statistics, the average probability of confusion (POC), over all of the pixels K of the region of interest, is:

$$\left\langle P\left(X_1 = X_2\right)\right\rangle = \frac{1}{K}\sum_{i=1}^{K}\sum_{x_i=0}^{\infty} poiss(\mu_{1,i}, x_i)\, poiss(\mu_{2,i}, x_i) \to \frac{1}{K}\sum_{i=1}^{K}\exp\left(-\mu_{1,i}-\mu_{2,i}\right) I_0\left(2\sqrt{\mu_{1,i}\mu_{2,i}}\right)$$

Above, $\mu_{1,i}, \mu_{2,i}$ are the expected photon rates for X_1, X_2 at the i^{th} pixel. The function $I_0()$ is a modified Bessel function of the first kind, zero order and $poiss\left(\mu_{l,i}, x_i\right)$ denotes the Poisson distribution with rate, $\mu_{l,i}$ and argument, x_i . We propose that minimizing the POC between X_1, X_2 will yield a phase mask that allows us to distinguish between their respective specra. To test this, we optimized a phase mask for the spectra of four commonly used organic dyes, simultaneously. This involved averaging the POC over all unique pairs $(l,m)=(m,l)$ for $\left\langle P\left(X_l = X_m\right)\right\rangle$, $\ni m,l \in [1,4]$. These spectra – Alexa Fluors 647, 660, 680, 700 [8] – have a high degree of overlap (Fig. 1.h). After minimizing the POC, the PSF showed a large improvement in correct spectral identification (Fig. 1.a) over an Airy spot (Fig. 1.b). In the future we will apply the above work to multi-color experiments using red dyes.

References:

[1] E Betzig et al., Science **313** (2006), p. 1642.
[2] ST Hess, TPK Girirajan and MD Mason, Biophysical Journal **91** (2006), p. 4258.
[3] WE Moerner, Journal of Microscopy **246** (2012), p. 213.
[4] JT Martineau et al., Microscopy and Microanalysis **24** (2018), p. 1364.
[5] Y Shechtman et al., Nature Photonics **10** (2016), p. 590.
[6] LEW Eran Hershko, T Michaeli and Y Shechtman, arXiv:180701637, (2018).
[7] J Broeken, B Rieger and S Stallinga, Opt Lett **39** (2014), p. 3352.
[8] ThermoFisher Spectraviewer, https://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html (accessed February 17, 2019).

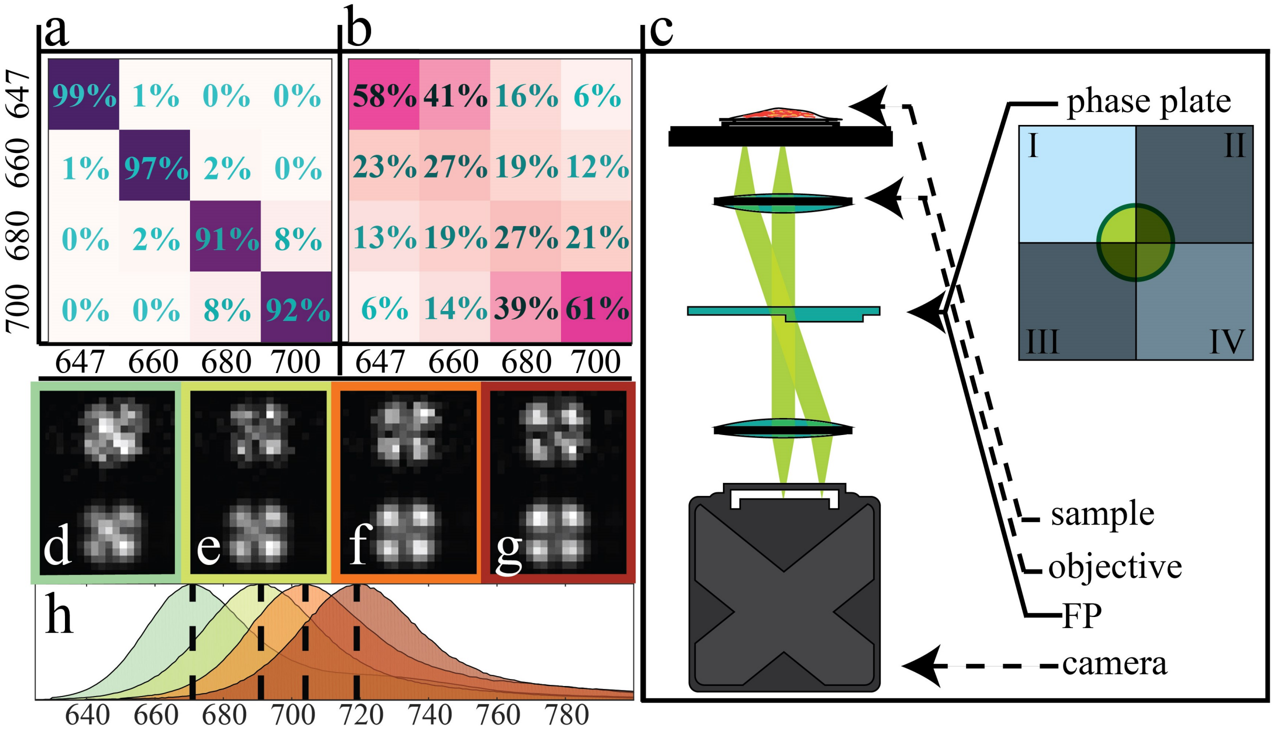

Figure 1. Panel (a): truth table (TT) for results of Monte Carlo spectral identification. Each PSF was simulated using a weighted sum of monochromatic PSFs calculated using a modified Born-Wolf model PSF. This sum was calculated using fluorophore spectra sampled every 10 *nm* (17 samples each).The PSFs were simulated with 1,000 photons. The horizontal axis denotes the true simulated spectrum for 1,000 trials and the vertical axis denotes the identified spectrum using a maximum likelihood fitting scheme. Correct identifications reside on the diagonal. For the optimal phase-mask, the rate of correct detection is above 90% in all cases. Panel (b): the TT for an Airy spot. Correct detection is 61%, at best. Panel (c): a simple diagram of the detection path of a wide-field microscope with a phase mask. The phase mask design we used in this analysis consists of four regions, I-IV, with an index of refraction of 1.46. For the optimized PSF the thickness of the four regions, I-IV, are as follows: 0 µm, 2.44 µm, 2.44 µm, 2.15 µm. Panels (d-g): simulated average optimal PSFs (bottom) and noisy optimal PSFs (top).The panels are arranged in order of increasing peak wavelength. This minimization was done using particle swarm optimization. Panel (h): the fluorophore spectra used in our simulations. Neighbouring spectral peaks are separated by between 10-20 *nm*.

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