Exploring Widefield Fluorescence Image Formation

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Abstract—Here I explore various aspects of widefield fluorescence microscope image formation. I focus on estimation of the point spread function (PSF) of one of my lab's microscopes under conditions similar to those I encounter in real samples. I use this empirical PSF for an initial attempt at deconvolution with poor results. But I've got high hopes for my next steps!

Index Terms—Computational Photography

1 Introduction

INVESTIGATION of minute biological structures requires the use of imaging modalities that can yield clean, interpretable 3D images. The laser-scanning confocal microscope is a workhorse of modern biological imaging. By scanning over a sample and removing out-of-focus light with a spatial pinhole, confocal microscopes confer a high degree of axial clarity, enabling robust 3D reconstruction of biological structures across many different scales. But these microscopes do have their disadvantages. The high sample illumination required can lead to phototoxicity and death of live specimens, and long imaging times can make it difficult to achieve high temporal resolution. Confocal microscopes are also quite expensive. Various sophisticated modalities (eg, light sheet microscopy, spinning disk confocals) can alleviate these concerns to a greater or lesser degree but are less accessible. Widefield fluorescence microscopy, by contrast, is a simpler system in which an entire frame is illuminated and recorded at the same time. A focus stack can be collected across the depth of a sample with a widefield microscope, but each slice is contaminated with a great deal of out-of-focus light (figure 1) making interpretation of the actual distribution of fluorophores in the sample difficult. Hypothetically, one can perform 3D deconvolution on these focus stacks to better estimate this distribution. Here I explore various aspects widefield fluorescence image formation, particularly estimation of the microscope PSF, as I try to achieve this.

2 Related Work

Deconvolution of widefield fluorescence microscope focus-stacks is a well studied approach for 3D imaging [1]. An essential first step is an estimation of the point spread function (PSF) of the optical system, which can be done theoretically or experimentally. [1], [2]. With an image formation model in hand various inverse methods can be employed to achieve deconvolution. Noise in the image formation process presents a difficulty and must be properly accounted for to avoid reconstruction artifacts. [3].

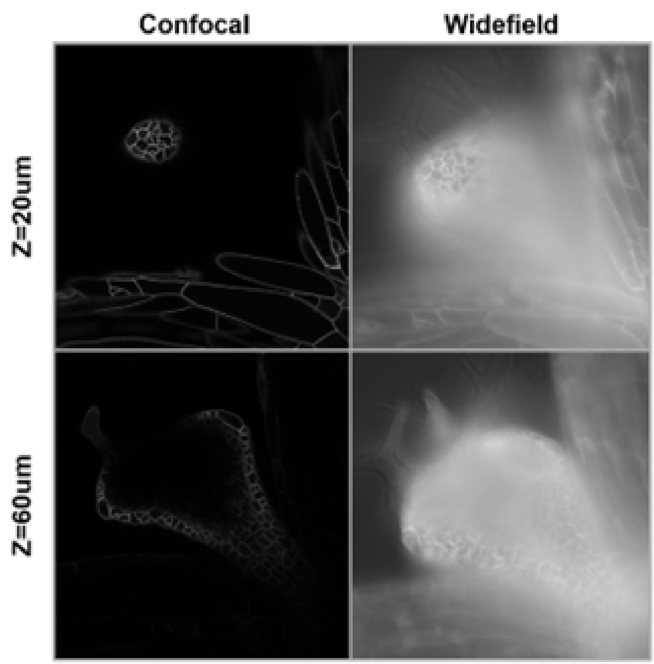

Fig. 1. Confocal and widefield micrographs of the same leaf, taken at two different focus planes.

3 Method

For this project I focused mainly on estimating the PSF of the widefield fluorescence microscope I intend to use for later experiments and applying this to some initial attempts at deconvolution. As my 'point' sources of light I used red fluorescent beads of a diameter below the diffraction limit of the microscope/objective. The Abbe diffraction limit is wavelength/2*numerical aperture. For red light (610nm) and a 0.8NA objective a diameter ¡ 300nm should be used. I used 200nm beads. I dried these beads onto a glass microscope slide and immersed them in about 300um of water below a glass coverslip to mimic the actual imaging conditions of my experiment. I also tried drying them onto an actual sample–a leaf–but this yielded messier images (chlorophyll emits in red). I collected 100um deep focus

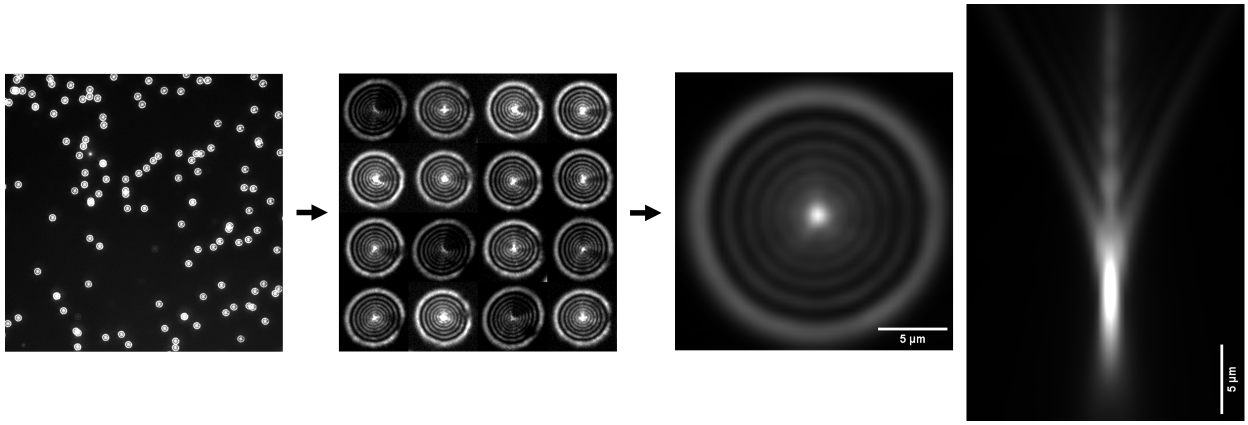

Fig. 2. Averaging bead images to get an empirical PSF. On left is image of scattered beads dried on a glass slide. These were averaged to yield the clean PSF on the right. Far left is 'side' (XZ, Z as axial dimension) view, to the right of this is the 'top-down' (XY) view.

stacks (0.5um/stack) of 2048x2048px images at 0.325um/px resolution. I then pulled out smaller 30x30x50um regions around 70 non-overlapping beads. I aligned and reinterpolated each bead image onto a reference via phase cross correlation and averaged them. I then recentered the image to place the brightest point in the center and normalized it to integrate to one. I took this as my empirical PSF (figure 2).

I then used this empirically estimated PSF to convolve and deconvolve some simulated 3D images. I convolved each image with the PSF and then added different amounts of gaussian noise before applying a wiener filter as an initial attempt at deconvolution.

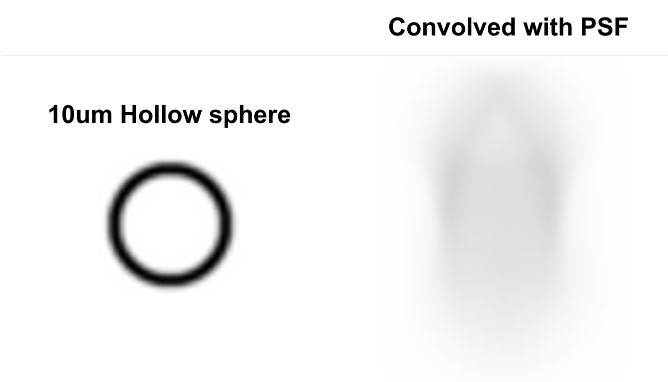

Fig. 3. Simulated 10um hollow sphere (XZ slice) and sphere convolved with PSF

4 Analysis & comparison

As an alternative to the described empirical approach, PSFs can also be generated from optical theory as in [2]. This theoretical approach has the benefit of being noise-free, but will miss aberrations specific to a given optical system that are not accounted for in the model. Averaging together many real PSFs also reduces much of the noise in the empirical approach. Here I also do not account for variability across the imaging plane. This is an issue, as the PSF at a point far to the edge is likely different from a point near the center. Some of this variability is evident in figure 2 panel 2.

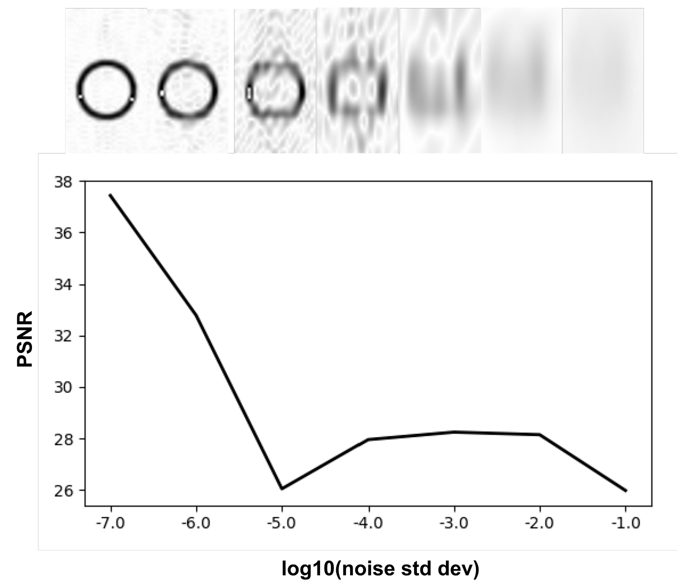

Fig. 4. Simulated 10um hollow sphere (XZ slice) and sphere convolved with PSF

5 Results

We see the shape of our widefield fluorescence microscope PSF to the right of figure 2. The observed shape matches what the expected theoretical PSF [2]. If there were no intervening layer of water between the beads and coverslip we would expect a more symmetric 'X' shape in the XZ plane. I'm unsure whether there is a significant depth dependence on the shape of the PSF below this already 300um thick layer of water. From the XZ perspective (figure 2 far left) we see how this PSF stretches observed objects in the axial dimension. This is quite evident in figure 3. I've simulated a 10um hollow sphere and convolved it with our PSF, yielding an approximation of what this sphere would look like imaged on a widefield fluorescence microscope. It is quite blurry and stretched.

In figure 4 I've applied a wiener filter to this convolved image after applying varying amounts of gaussian noise. At very low levels of noise it performs well enough, but as the amount of noise increases the PSNR of the reconstruction rapidly decreases. Unsurprisingly, this naive approach does not perform well on my real widefield images and instead introduces messy artifacts.

6 Discussion

Clearly I need to go about deconvolution in a more sophisticated way using some of the techniques we learned about in class. My next step will be to implement ADMM with a few different regularizers. I'm not sure exactly what prior best fits my samples. Whereas a TV regularizer works well for natural images, which are often approximately piecewise continuous, I'm not sure this will work well for my samples. [3] uses the Frobenius norm of the Hessian of the image. I still have some work to do before I can get a good reconstruction.

References

[1] McNally *et al.*, *Three-Dimensional Imaging by Deconvolution Microscopy*, Methods 1999

[2] Kirshner *et al.*, *3-D PSF fitting for fluorescence microscopy: implementation and localization application*, Journal of Microscopy, 2012

[3] Ikoma *et al.*, *A convex 3D deconvolution algorithm for low photon count fluorescence imaging*, Scientific Reports, 2018