Three Dimensional Deconvolution of a Confocal Microscope

William Kyle[1] and Jan Kmetko[1]

[1]Department of Physics, Kenyon College, Gambier OH, 43022

Introduction

Due to the application of new methods of light based microscopy, and through general improvements in the precision of manufactured optical components, the imaging capabilities of visible light microscopes, and confocal microscopes in particular, have reached the point at which they are capable of such precise imaging on small enough scales that they are only limited by inherent properties of diffraction that belong to the light itself. What this means, is that light based microscopy has reached a fundamental limit in resolving power which, as a product of the medium of light through which they operate, means that no enhancement of the design of the microscope will ever result in the ability to resolve objects smaller than what they are already capable of. As a result, alternative methods which operate independently from the microscope's method of imaging are required for sampling at smaller than the diffraction limit of the microscope. This is the power of deconvolution as a tool in microscopy. By creating a deconvolution algorithm for our confocal microscope, we hoped to not only improve detail in images beyond our microscope's diffraction limit, we also hoped to be able to recover information about samples that had been completely unresolvable in the unprocessed images.

Theory of Deconvolution

In it's most bare essence, deconvolution is a process which allows us to use our knowledge of the physics of optics and the propagation of light to correct images from our microscope of some of the blur produced by light diffraction within the microscope. More specifically, the way that light is imaged through a microscope is a process which we can model as a convolution operation.

$$s(t) = h(t) * u(t) \quad \stackrel{\text{def}}{=} \int_{-\infty}^{\infty} h(t-\tau)u(\tau)d\tau$$

(Where $*$ is the convolution operator, giving a superposition integral)

However, this equation ends up being extremely difficult and time intensive solve using available computational methods. So, this definition only serves to provide some intuition about what is happening in the convolution of a signal. Instead, I worked with convolutions using the FFT Method. This method converts the $*$ operator into a regular multiplication by transforming $s(t)$, $u(t)$, and $h(t)$ into frequency space by taking their Fourier Transforms.

$$S(f) = H(f)U(f)$$

(Capital letters denote Fourier Transform)

What these equation describe tells us how a measured signal $(s(t))$ can be described as a ideal, uncorrupted description of a sample $(u(t))$ that has been smeared out and modified according to a function $(h(t))$. In the context of microscopy, this means $s(t)$ is the raw image taken of the sample, $u(t)$ is a perfectly imaged representation of the sample, and $h(t)$, which is known as the Point Spread Function (or PSF), describes how the image is smeared due to diffraction of light in the imaging process.
This Point Spread Function is what we need to be able to deconvolve images from our microscope. Once we have a close enough approximation to the Confocal PSF, we can use H and S to deconvolve the raw image back to something that is a closer to u(t) than the raw image was. This is described by the equation,

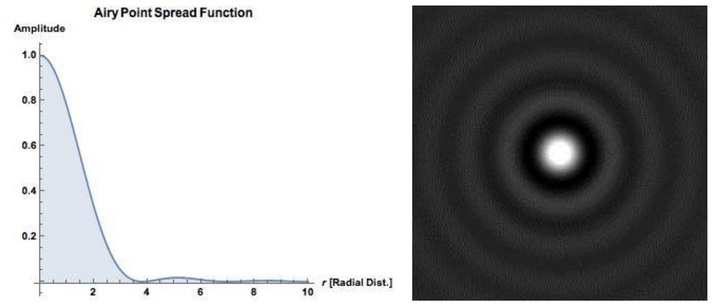
$$\overline{U}(f) = G(f)S(f) \qquad G(f) \equiv \frac{1}{H(f)}\left[\frac{|H(f)|^2}{|H(f)|^2 + \epsilon}\right]$$

(The $\overline{U}(f)$ term implies that we can only find an approximation of $U(f)$)

Here, $G(f)$ is our Weiner Filter which is what relates to the PSF we have calculated. It accounts for the fact that there is a baseline of noise within the image that is inherent to real world imaging. This noise is impossible to eliminate, but by adjusting our variable epsilon in $G(f)$, we can adjust the Signal to Noise Ratio being accounted for by the filter. Thereby, we are able to minimize the effect of the noise on our deconvolved image.

Formulating The Confocal PSF

In terms of the transverse imaging mode of a microscope, the most general form of PSF is described by an Airy Function:

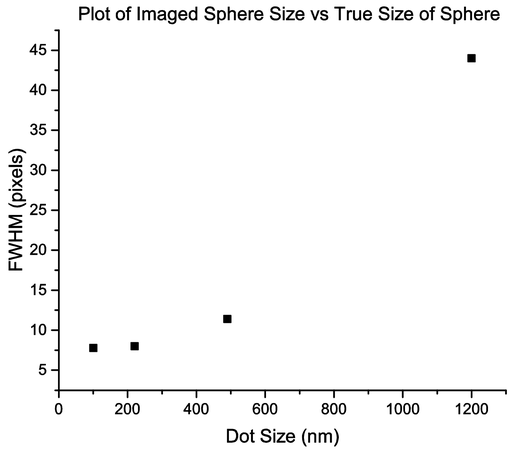


This PSF can be applied to almost all forms of optical arrays, that are designed to use a certain wavelength of light to image that sample. Therefore, we used this PSF as a proof of concept on our microscope by imaging through it in the wide-field mode. However, in the confocal imaging regime, this PSF is no longer accurate for two reasons. Firstly, the design of our confocal microscope uses both a confocal pinhole, and 2 separate wavelengths of light for imaging, both of which mean that it uses optical principles which don't conform to an Airy function PSF. Secondly, we want to deconvolve images, not only in the transverse mode, but also in the axial imaging mode. Therefore, it was to necessary to create a non-paraxial approximation of the Confocal PSF from scalar Debye theory.

$$PSF = |h_{ex}(x,y,z)|^2(|h_{em}(x,y,z)|^2 * P(x,y))$$

Methods

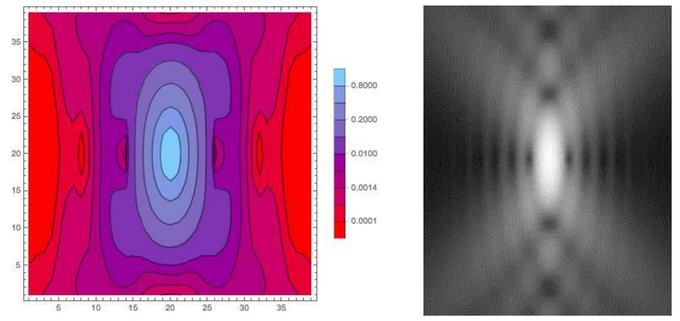
First of all, we needed to be able to measure what the diffraction limit of our microscope is so that we could understand the current limits on our microscope. This understanding enabled us to know within what regimes our microscope could be trusted as accurate in taking measurements I required to design a PSF.



This allows us to see that as we expected, the measured width of the calibration spheres decreases linearly up until a sphere size of around 250nm. Therefore, this is roughly the diffraction limit of our microscope in the transverse imaging mode, and so this is the imaging limit which we hope to overcome through deconvolution.
Secondly, since the image of a spherical sample asymptotically approaches the PSF as the size of the sample gets much smaller than the diffraction limit, this graph also tells us that the Full Width Half Max (FWHM) of our microscope will be 7 pixels. This is crucial because the FWHM is a characteristic which doesn't depend upon the type or classification of function to which it is being applied. Therefore, for any choice of function for our PSF, we can make sure it is properly scaled by calculating its FWHM and comparing it to the 7 pixel value we found. On top of this, I also prepared sample slides 1200nm spheres in a lattice, and using ImageJ, I measured center-to-center distances btw spheres and found a precise scaling factor of 37.5 nanometers per pixel.

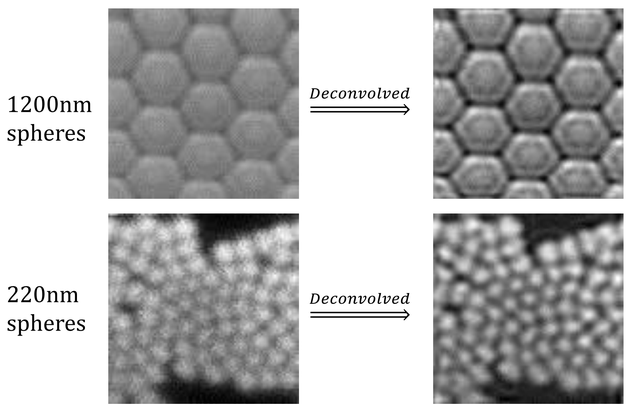
Results

Using the methods described, I was able to generate the follow PSF for our confocal microscope.

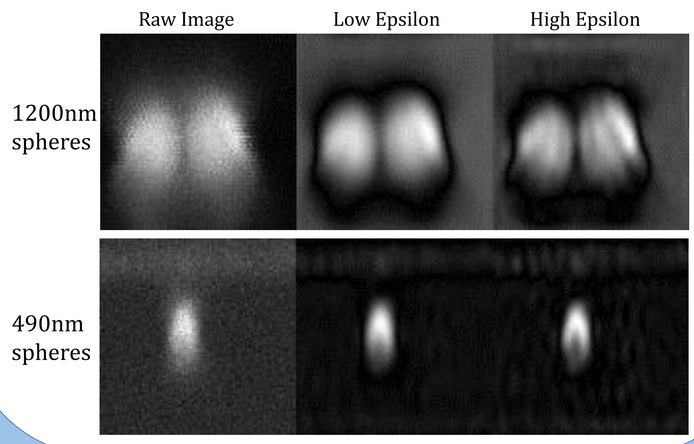


From this PSF, we were able to achieve the following deconvolutions with our microscope.

THE TRANSVERSE PSF



THE AXIAL PSF



Conclusions

The results we were able to get from our theoretically generated PSF ended up being very promising. Given that we had found a diffraction limit of 250nm for transverse imaging, and of 530nm for axial imaging, we were able to resolve details in samples that were smaller than these limits. In the image of the 220nm lattice in transverse mode, we can see much more definition in the location and shape of spheres than before convolution. Also, we were much more excited about the Axial deconvolution, because it enabled our discovery of uneven staining in our 490nm spheres. This caused a "dimple" feature in the middle of the sphere's location because they emitted less light from their internal volume.

Acknowledgements

[1] Dusch, E., et al. Journal of Microscopy (Oxford): Three-Dimensional Point Spread Function Model for Line-Scanning Confocal Microscope with High-Aperture Objective. 228 Vol. Blackwell Publishing, 2007. Web. 14 Oct. 2015.

[2] Press, William H.; Teukolsky, Saul A.; Vetterling, William T.; Flannery, Brian P. (2007). Numerical Recipes in C: The Art of Scientific Computing (3rd ed.). New York: Cambridge University Press. ISBN 0-521-43108-5.

I would also like to thank Professor Kmetko and the Kenyon Physics Department, as well as the Kenyon Summer Science Scholars program, for making it possible for me to complete this project.