Point Spread Function Workshop
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Introduction

In an ideal world, we would be able to use microscopes to resolve objects of any size, from millimeters all the way down to nanometers (which would be super awesome when we try to look at the insides of a cell). Unfortunately, life is not so kind. The phenomenon called diffraction, which refers to the bending of light when it encounters small objects and the spreading out of light when it goes through a small opening, prevents us from achieving unlimited resolution. A good rule of thumb to keep in mind is that the limit diffraction places on the resolution of a microscope is given by the equation

$$d = \frac{1.22\lambda}{NA}$$

where lambda is the wavelength of light, NA is the numerical aperture of our objective, and d is the resolving power of the microscope - i.e. the size of the smallest feature we can resolve. In this exercise, we will gain some intuition as to why this is the case.

Suppose we have an object smaller than the resolution of our microscope, say a fluorescent bead 200 nm in diameter. When we image this object, we excite it with a light source and then use optics to guide the photons to our camera to collect them. We can think of this bead as a point source for photons. Ideally, when we look at the image where our camera took, we would see a single point at the exact location of the bead. This is not the case - in reality, the photons spread out and what we see on the picture is a smear, localized around the location of the bead. This smear, or rather the response of our microscope to a point source of photons, is called the point spread function.

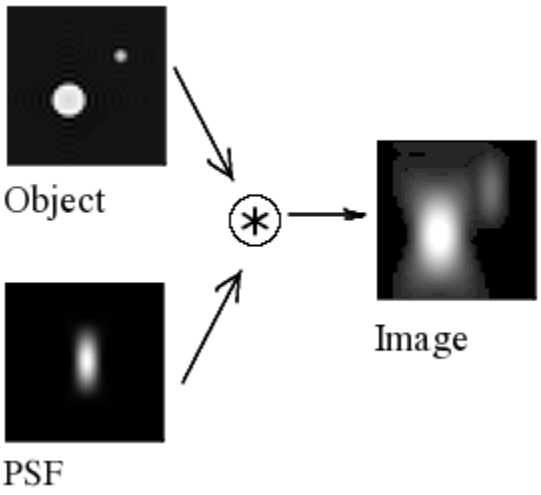

Figure 1: A point spread function. The image that we capture with a microscope is a convolution of the actual object with its point spread function. Image taken from wikipedia.

The response of our microscope to an arbitrary source (or arbitrary object) is then the convolution of the object with the point spread function (or PSF). We can start to see how this will limit our resolution. If we have two objects really close to each other, after the convolution with PSF, they might be smeared together and look like one object on the screen.

Measuring the PSF

In this lab, we will measure the point spread function of a microscope by looking at beads 200 nm in diameter, smaller than the resolution of the microscope.

1. Assemble a small flow chamber by placing two pieces of double sided sticky tape on a glass slide and placing a coverslip on top.

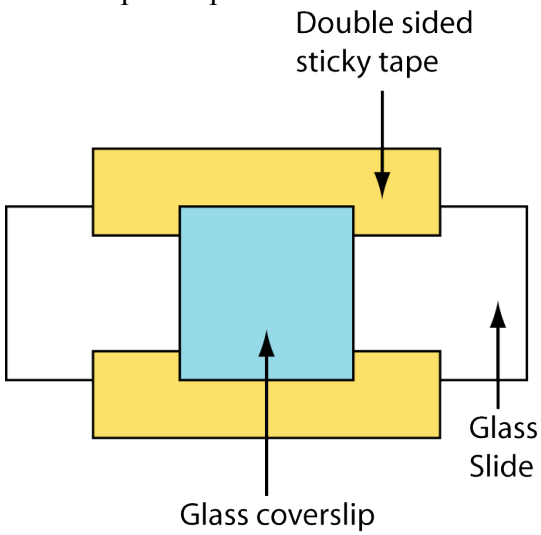

Figure 2: Assembling a simple flow chamber.

2. Create a 1:10,000 dilution of fluorescent beads into distilled water. Pipette about 20 uL of your bead solution into your flow chamber and turn it upside down (coverslip side down). After a couple minutes, wash the chamber with 100 uL of water to remove the beads that haven't stuck to the surface. Seal the chamber with nail polish

3. Image your sample at 100X in fluorescence using the FITC filter set. If you are having trouble focusing, try focusing on the tape and then moving into solution. Once you have see some beads, adjust the z-position of the microscope so that the beads are as small as possible (it usually helps to have the exposure time set to about 200 ms during this step).

4. With the beads focused, use the multi-dimension acquire feature in micro-manager to collect a z-stack of images of the beads. The acquisition should be set to "relative z"; 2 microns above and below the plane of focus is probably sufficient. Try to use the smallest step size possible - for the Nikon microscopes this is 0.1 microns.

5. Copy your images to snowdome.caltech.edu so you have access to them later.

Visualizing the PSF

Now that we have collected some data, we can use MATLAB to visualize the microscopes point spread function. The following two sections can be done at home at your leisure (as long as the finished product is handed in by the due date). You will be required to turn in two things - a commented MATLAB m-file that performs the tasks below and a website containing the pictures you create along with some commentary.

We want to visualize the PSF in the xz plane (how the point source spreads out as we move away from the focal plane) and the xy plane (how the point source spreads out in the focal plane). Write a m-file that does the following

1. Load the images you obtained of fluorescent beads into MATLAB's memory. For each frame, crop out a rectangular box surrounding one particular bead and save the cropped image as a variable. Because you might have 30 or more images, it might be useful to save the cropped image in a 3-dimensional array. For instance, you could save the cropped image from the first frame in the variable `I(:,:,1)`, the image from the second frame in `I(:,:,2)`, etc.

2. Next, we want to take a cross-section of the bead for each frame and assemble it into its own image. This will be how we visualize the xz PSF. Using imtool, find the coordinates of a row that goes through the center of the bead. This is schematized below

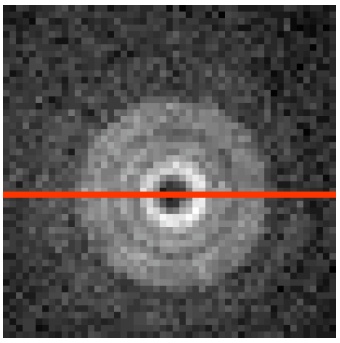

Figure 3: Taking a cross-section of one frame.

For each frame, extract the pixel values in this row, and make this a row in a new matrix. For instance, if the 30th row goes through the center of the bead, one would take `I(30,:,1)` for the first frame and store it as a row (or column) in a new matrix.

3. Make a picture of the xz PSF.

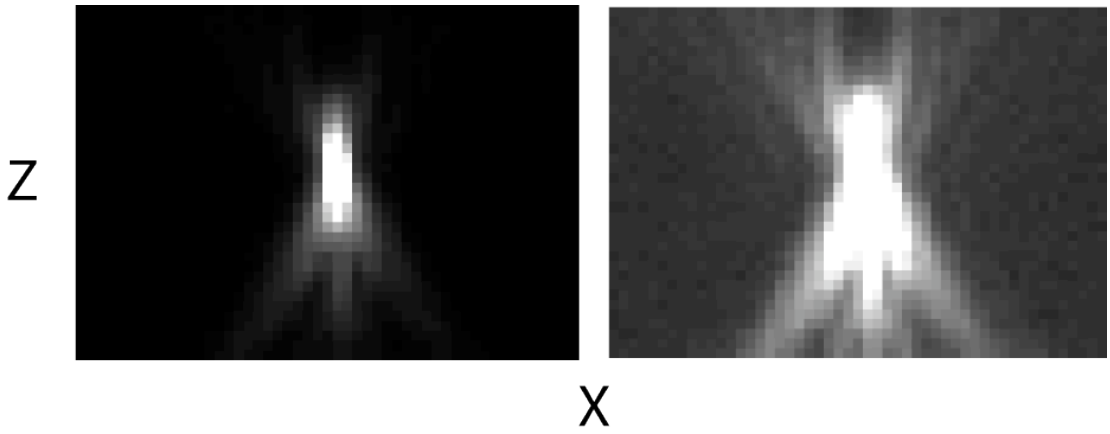

Figure 4: A picture of the xz PSF. Both pictures have been contrast adjusted.

Toy around with the contrast so you can get a feel for its structure. Be sure to label which axis corresponds to the x-coordinate and which axis corresponds to the z-coordinate. Comment briefly about how the PSF changes when you move in and out of focus and how this will affect your image quality. Also comment about how far physically from the focal plane you have to be before you expect your image quality to be severely degraded.

Next, we want to visualize the xy PSF - this is what limits the spatial resolution of our microscope. The PSF of our beads should be an airy function - in this exercise you will

1. Select the frame that is closest to the focal plane. This is usually done by picking the frame where the fluorescent bead looks the smallest and is the brightest.

2. Assuming our microscope is diffraction limited, one can show that PSF is expected to be an Airy disk. The formula for an airy disk is given by

$$I = I_0 \frac{J\left(\frac{r}{r_0}\right)}{\frac{r}{r_0}}$$

where J is the first bessel function, r is the distance from the center of the peak, I_0 is the intensity and r_0 is a length scale related to the properties of the microscope and the wavelength of the imaging light. Using MATLAB, perform a nonlinear least squares fit of your xy image to a 2-dimensional Airy function. A few things to keep in mind while you are doing this:

    1. You will need to write down a cost function to be minimized. This is probably best done as a separate function - see the users guide or one of your TAs if you don't know how to write MATLAB functions
    2. Your fitting parameters will likely be I_0, the location of the peak, and r_0. Also, think about how you will account for the background. You might want to make it another fitting parameter (in which case $I = I_0 J(r)/r$ +background) or you may

want to estimate it another way and subtract that value from all the pixels. Be explicit about which one you chose and why you chose it.

3. The MATLAB functions fminsearch and besselj(1,r) will probably be helpful.

3. Using mesh or surf, make a plot of your actual data and your fit and place them side to side to compare. Make sure the axes have the same scale so you can make a fair comparison. Comment on the goodness of fit.

4. The Airy disk can often be approximated by the 2-dimensional gaussian

$$I = I_0 \exp\left[\frac{(x-x_0)^2+(y-y_0)^2}{r_0^2}\right] + B$$

Repeat your least squares fit using the two-dimensional gaussian instead of an Airy disk. Again, be explicit with how you determine the background and make pictures comparing the gaussian fit with the actual data. Make a plot of how the Airy disk fit and the Gaussian fit depends on the distance to the center. Are there any features of the Airy disk that the gaussian fit can't capture?