TIPS FOR BETTER CONFOCAL IMAGES:

Strategies to fight the battle of signal vs. noise.

Ideally, *if signal strength were not a limiting factor*, we would use the following options:

- very low laser power and quick scan speed to protect the sample
- a small pinhole to create thin optical sections
- a narrow wavelength window to reduce detection of bleedthrough signals from other fluorochromes
- low gain on the PMTs to reduce noise (signal variability that is exaggerated by amplification)

Unfortunately all these choices reduce the signal, resulting in a lower signal/noise ratio. To boost signal, you must sacrifice in one or more of these areas.

Changing each variable is a tradeoff: the upside and downside are described for each. They are listed in order of desirability for typical situations. These tips work best if you have already optimized your sample as well as the optical path (make sure the objectives are set up properly).

a. Open the pinhole. The "Airy 1" pinhole size is the default setting, but this default is the optimum for creating the thinnest possible section, *not* the brightest image. Your current pinhole setting may be too narrow to allow sufficient signal to pass through. Open pinhole control window and increase pinhole size with the slider. Compare image quality before and after to ensure that important image features are not degraded.

Good: does not increase bleaching or wavelength range.

Bad: confocal slice gets thicker in the Z direction; more out-of focus light is detected (depending on application, this may not be a problem).

b. Widen the wavelength range detected. The more of the emission you detect, the brighter the image.

Good: does not increase bleaching or the depth of the confocal slice.

Bad: you may start to detect fluorescent sources other than the desired one.

c. Try different laser line and dichroic combinations. Maybe your fluor is not behaving as predicted and another combination will work better.

Good: no downside if it works.

Bad: rarely works.

d. Increase gain on PMT from 500 (default) up to ~700. 800 is the maximum-don't go higher! You may need to readjust the offset as well, to eliminate any "0" pixels.

Good: does not increase bleaching, the wavelength range, or the depth of the confocal slice.

Bad: noise is also increased. We find that areas of uniform signal show greater pixel-to-pixel variation as the gain is pushed up over 700. This false (nonreproducible) signal variation can be smoothed using the basic "Blur" function in Photoshop, generally without any loss of true resolution.

e. Increase laser power/dwell time. The more power you pump into the sample, the more emission you will get out (though this effect plateaus at some point, depending on the fluorochrome). The % laser power can be raised in the Beam window. If you reach 100% and need more, AS A LAST RESORT, the dial on the *laser power* box can be turned clockwise. This dial only affects the power on the 458/476/488/514 laser. The scan time can also be slowed, effectively increasing the power delivered to the sample.

Good: does not increase the wavelength range, or the depth of the confocal slice.

Bad: Fries your sample. Both bleaching and sample damage increase substantially as laser power goes up. You can test bleaching time using an unimportant area of the sample (Qdots should not bleach). If you see rapid bleaching, make sure you use an antifade, and that the antifade is not overly diluted (we use Vectashield). Sample damage is harder to assess; you might check viability of live samples after exposure to different laser levels.

f. Decrease image size (pixel number), for example from 1024x1024 to 512x512. This makes the pixel size bigger, so more signal goes into each pixel.

Good: does not increase bleaching, wavelength range, or the depth of the confocal slice. Also, makes file sizes more manageable.

Bad: The larger the pixel the lower the resolution. If you later need to zoom in on part of the image, you will find it pixelated (jagged). This may not be a problem if low-res images suffice for your application, but there is no way to bring back the lost resolution later on.

g. Photoshop it. Collected images may be stretched to cover the range of gray values, and contrast may be altered (be sure to *save as*; don't save over the original file!)

Good: simple way to fix images after collection.

Bad: there is no perfect way to separate signal from noise, so both get stretched. Stretched images tend to look grainy. Remember, Photoshop can't create data that is not present in the original file.

You may want to collect TWO scans of each important image, then average them in Photoshop. This significantly reduces graininess when the image is zoomed or stretched.

Kevin Edwards, 2007 (kaedwar@ilstu.edu)