LEICA SP2 CONFOCAL TIPS.

<u>Ways to increase signal</u>.

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

- very low laser power and quick scan speed to preserve the sample
- a small pinhole (Airy 1) 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 (random 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.

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, and typically gives a very dim image. 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 (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 signals 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) toward ~700v. Do not exceed 800v. 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. Areas of uniform signal will show increased levels of pixel-to-pixel variation as the gain is pushed up over ~550.

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, the dial on the *laser power* box can be turned clockwise. This dial only affects the power on the 458/476/488/514 laser lines. The scan time can also be slowed, effectively increasing the power delivered to the sample.

Good: does not increase the noise, 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. 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). This makes the pixel size bigger, so more signal goes into each pixel.

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

Bad: You may be undersampling the available optical resolution. If you later need to zoom in on part of the image, you will find it pixelated. This may not be a problem if low-res images suffice for your application, but there is no way to rescue the lost data later on.

g. Photoshop it. After image collection, the pixel values can be uniformly stretched, so that the image uses the full range of gray values. Any alterations must be done uniformly on all pixels; never make alterations on just a selected region of the image. Even if the alteration is uniform, it must be well documented, and follow the rules of the journal if the work is for publication. Be sure to *save as*; don't save over the original file! See Photoshop handout for details.

Good: can be done post-collection, and can sometimes "rescue" important features of the image that are hard to see in the original.

Bad: it is hard to separate signal from noise, so both get stretched. Stretched images tend to look grainy. Remember, Photoshop can't create real data that is not present in the original file.