Improving Image Quality on the CMI confocal systems

Some days it is difficult to come up with a brightly stained sample. This document contains some strategies to improve your image quality. None of these will take a crap sample and make it worthy of the cover of SCIENCE or NATURE, but you may find that you can get a pretty decent image if you work at it. These strategies might give you enough information to go back to your lab and fine-tune your staining parameters. Remember that the quality of your images is <u>significantly</u> affected by your sample preparation.

Confocal microscopes are inefficient with light, which means that they work best with bright samples. If you really have to stare through the microscope eyepieces to see your sample (or need to turn the room light off), your sample is most likely not bright enough to give a good-looking confocal image.

Note: This document assumes that your sample is either fixed cells or tissues. Working with live cells/organisms on the confocal is a careful balance between avoiding photo-toxicity and getting nice images. Sometimes the best live-cell data <u>has</u> to be noisy to avoid damaging the cells.

To make this all work we will need to understand and possibly make some decisions about which aspect of physics/engineering we are willing to trade off to get what we want/need. There is no "free lunch"; you may have to sacrifice something to get what you want.

The Imaging Triangle

Users have to decide the balance between the competing needs of time resolution, spatial resolution and intensity resolution to obtain the best results for their needs. Unfortunately, you can usually only have two out of the three corners of the triangle.

Time resolution

Scanner speed, averaging,sampling…

Spatial (XYZ) resolution
Pinhole size , pixel resolution,sampling (x,y, &z), objective NA….

Intensity/sensitivity resolution
Detector gain/offset,signal intensity,averaging, scan speed, Objective NA
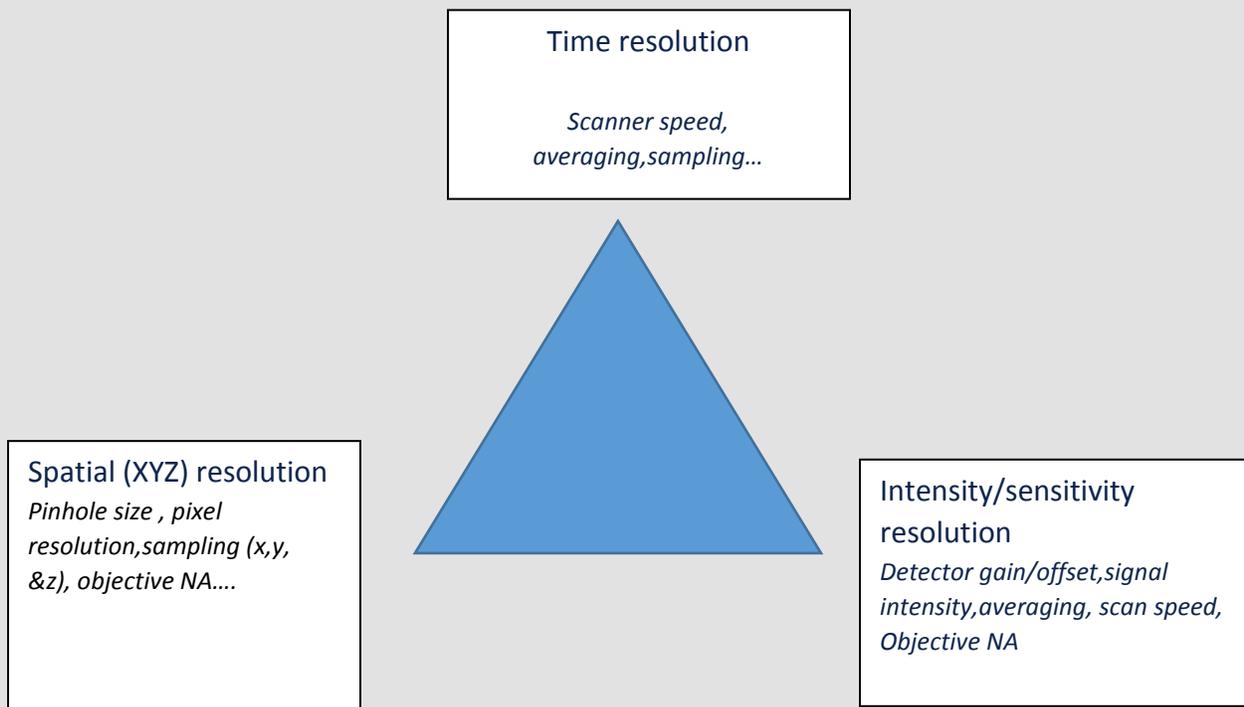
How big should your image be?

The Olympus FV3000 can capture images at image sizes ranging from 512x512 pixels to 4098x4098 pixels, z slices at 0.1 μm steps, and in time as fast as 30 FPS@512×512 and 438 FPS@512×32. Note, just because the instrument has these capabilities, does not mean that you "need" them in every instance.

Issues related to image size include:

- Time – it takes additional time to capture more pixels/z slices/time points in the same field of view.

- Recording the optical resolution – if you have items in your specimen that are at (or near) the optical resolution of the microscope (0.3-0.4μm lateral or XY), mathematical theory (Nyquist/Shandon) says that you need at least 2.5 pixels in each direction to correctly "sample" the information without introducing artefacts (see example on the next page). The best image size (assuming the pinhole is set to 1 Airy Unit) will differ depending on the lens you are using and any zoom being used, since optical resolution is a mathematical function of the numerical aperture (NA) of the lens and the wavelength of the laser. NOTE: See the table at the end of this document for calculated "best" pixel resolutions (meets sampling criteria) using different lenses and common zoom factors.

- Z stack sampling – In a perfect world, Z stacks would be correctly sampled at 2-2.5 times the effective Z resolution (see table on last page) as well. If you want to create a 3D rotation movie, I highly recommend using correct sampling in Z, as this will improve the quality of your movie.

- Live cells/time sampling – The same sampling theory applies to capturing moving objects in live cell imaging. You want at least 2-2.5X as many time points as the fastest movement in your sample. If this is not done, the artifact can easily be interpreted as a real data.
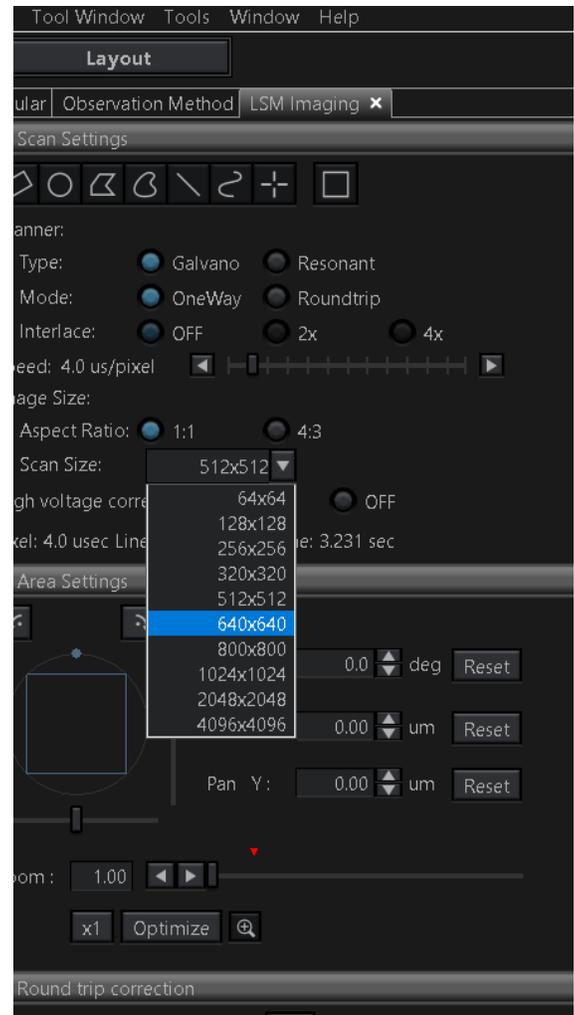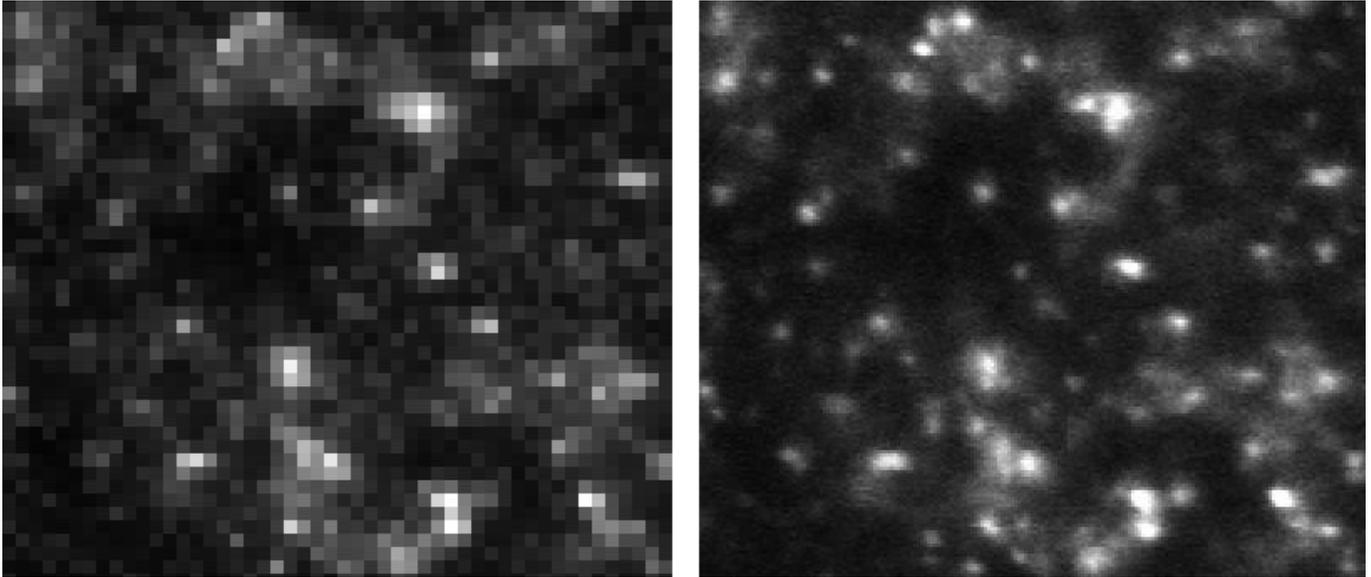
Insufficient number of pixels leads to sampling artifacts:

The left image was captured at 512x512 pixels, which is insufficient resolution and blurs adjacent in situ hybridization spots into pixelated 'blobs'. The right image was captured at 2048x2048, sufficient to allow the viewer to accurately count the in situ spots, within the same field of view.

Do I really need all that data? Maybe, it depends on how you plan to use the image.

- If you are looking for the presence or absence of whole cells (15-30μm in size) or other large objects, then you don't need to worry about resolving the tiny things in the image.

- Remember though that journals want their images at 300dpi, which means at this dpi resolution a 1024x1024 image is 3.4 inches across and a 512x512 image is only 1.7 inches. You can always reduce the number of pixels to use an image for publication or crop it to keep the size down. Digitally enlarging an image tends to make edges become blurry and does not add any additional optical resolution to the image.

Getting the most out of the Photomultiplier Tube (PMT, detector)

Source: http://learn.hamamatsu.com/articles/photomultipliers.html
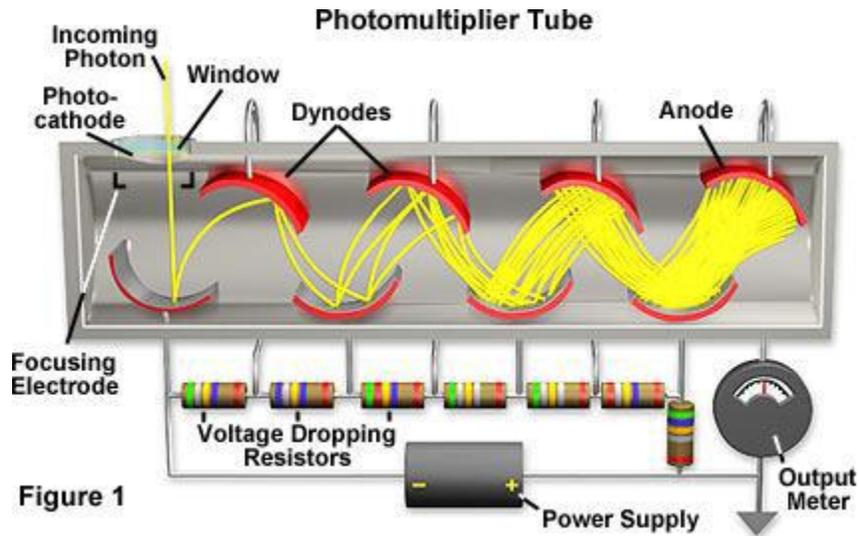
Confocals do not use a camera. The focused laser spot is scanned point-by-point across the sample and the signal from each point is measured by the detector (PMT). The detector's analog signal is converted to digital to give the intensity value for each pixel.

To detect the signal, a photon is converted to an electron at the PMT's photocathode window. This conversion is only about 25-30% efficient, so a great deal of light is lost here.

This conversion of a photon into an electron is also one of the important sources of noise in the image. Poisson statistics say that the noise will be equal to the square root of the signal. For example:

# of photons detected	Square root	Range of possible detected signals (1st standard deviation)
25	±5 photons (±20% of total)	20-30
100	±10 photons (±10% of total)	90-110
1000	±31 photons (±03% of total)	969-1031

Inside, the PMT is a vacuum. There is a charge differential such that the electrons are attracted to the back of the PMT. As the electron moves towards the back it encounters the dynodes, which are used to amplify the signal from a single electron to many electrons. The output meter measures the electron signal and sends it to the analog to digital converter to create the value for that pixel. The detector gain control on the confocal affects the amount of amplification that occurs, and the strength of the detected signal. The detector offset is the value

where the computer ignores the low intensities and just calls the signal black (intensity = 0). Higher detector gain settings may amplify random thermal noise in the detector and/or other electronic noise sources.

Adjust confocal pinhole aperture , or keep on auto

Sequential scanning

Detectors 2 HSD – high sensitivity

2 SD detectors – standard PMT's

All 4 spectral ranges are adjustable

1. Slow down your scans to reduce noise – Scan speed is determined by the speed with which the mirrors move the focused spot across the sample. The speed is expressed in µs/pixel, with higher numbers being slower.

   - Slower scan speeds give improved signal to noise ratios at the same detector gain settings. Since the spot spends more time in one place, it can collect more photons and reduce the Poisson (Shot) noise.

   - The downside is that slower scan speeds also increase the total light exposure of the sample, possibly leading to somewhat faster photo-bleaching. Live cells should not be exposed to large amounts of light, since photo-toxicity can occur. **Note that 405nm is a near-UV wavelength. Use of 405nm on live cells is not recommended as UV exposure has been shown to cause DNA damage.**

   - Slower scan speeds affect how long it takes to acquire a single image. If you care about the quality of an image, taking 30 seconds per channel is not unusual (=2min for 4 colours). This can be too slow for time course or Z stacks.

   - HV correction adjusts the detector setting in relation to the scan speed chosen
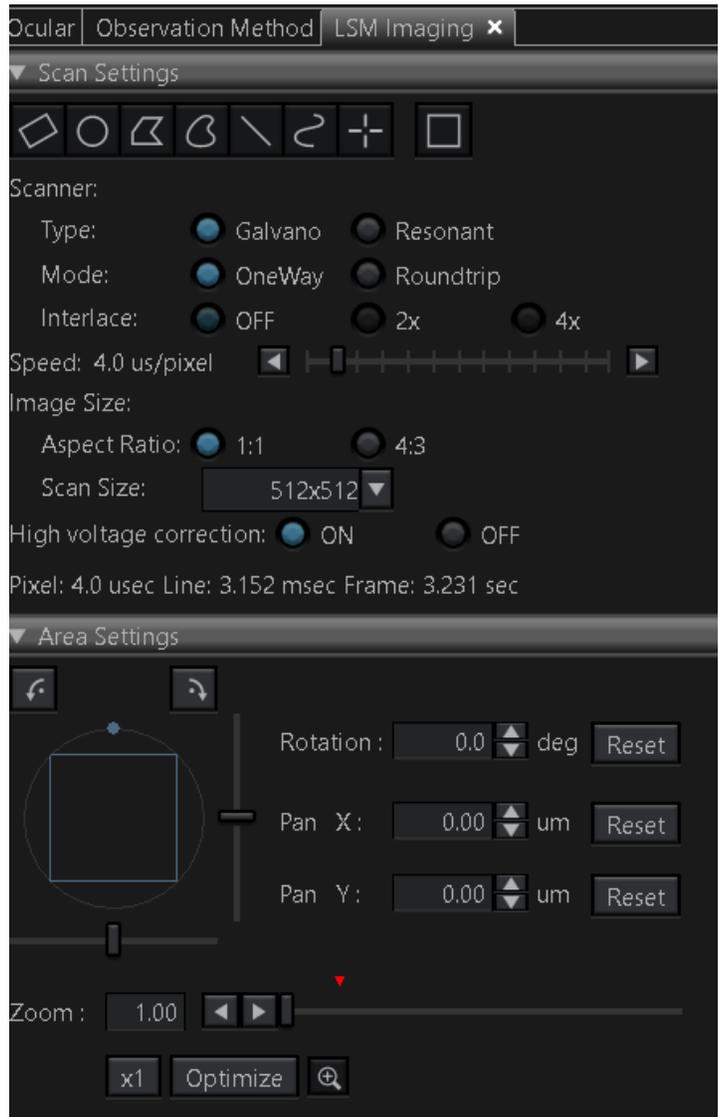
2. Lower detector voltage and gain gives the cleanest (lower noise) images – The PMT detector gain setting increases the fluorescence signal by causing each electron created in the PMT to be multiplied before it is measured. Higher gain values equal a brighter signal with an increase in electronic noise in the image. If you can adjust some of the other instrument settings so that this value goes down, your image will usually look "cleaner" (less noisy).

   - Once you increase the gain setting to above 800 the noise level is usually noticeable.

   - Make sure that you use the HI-LO (image window) to ensure that you do not saturate your image. BLUE colour indicates background. WHITE indicates saturation. A very tiny (occasional clusters of 4-6 pixels) amount of pure white is usually acceptable.

   - In some fields of view you may have debris or a structure in the image that is significantly brighter than the rest of the sample. You may choose to deliberately over-saturate these items to enhance the dimmer items in the image.

- DO NOT over-saturate important scientific image data just to make your sample look brighter; this is not good science.
- See Averaging for ways to reduce the gain and the noise.

3. Slight adjustments to the offset will ensure that your background is dark – This tells the PMT what the cut-off point is for black (no signal). If the electronic signal is below this setting, the pixel on screen will be displayed as black. Careful adjustment of this setting can improve the contrast in your image.

   - For biological samples, this is often 1-2% or just slightly negative of zero percent. It doesn't take a lot of adjustment to achieve the affect that you need.

   - Aggressively adjusting the amplifier offset is not the way for you to "clean up" your background. Biology and confocal images have electronic noise and perfectly black backgrounds do not exist. A perfectly black background could be perceived as inappropriate image manipulation.

   - It is also possible to adjust the amplifier offset to such an extreme that the dimmer parts of the image begin to completely disappear (they also are relegated to black). Use the HI-LO LUT to check your settings. You should only have very small amounts of BLUE (pure black) in your image. Small amounts means a speckling of BLUE (like stars in the sky).

4. Careful use of averaging can reduce noise – Noise is random, and signal tends to be more consistent. Averaging can help reduce noise. Remember that any of the averaging settings increase the overall exposure of your sample to the laser and increase the time it takes to acquire an image or images.
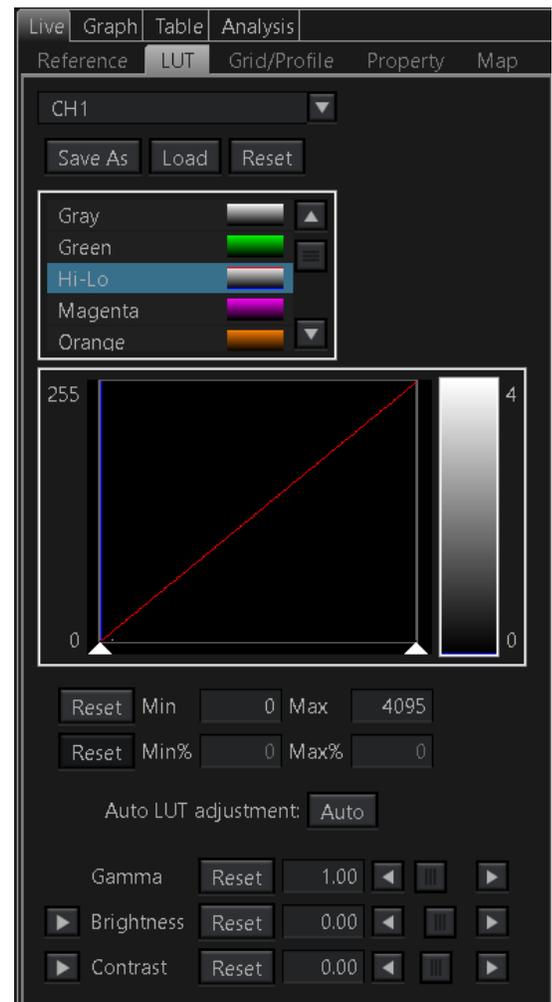
   The confocal scans the sample in a raster pattern (see figure). One mirror controls the spot's position in X (left to right), with a second mirror (Y) used to position the spot on a new line.

   Signal to noise ratios can be improved in a number of ways.
   - Line averaging – each line is scanned several times before moving to the next line, and the data for each point is averaged together to reduce noise.

   - Frame averaging – an entire image frame is scanned multiple times and then the data for each point is averaged to reduce noise. Because the changes made by frame averaging are not obvious (unless you look closely at the monitor), you will need to be patient and allow the instrument time to finish the averaging.

Averaging notes:

- Line averaging should be used if live cell imaging is being performed, since it is the fastest form of averaging and the least sensitive to movement.

- Given a choice between line and frame averaging, I would choose line. During line averaging only one scan mirror moves while the data is being collected, avoiding any re-registration issues that might occur in frame averaging.

- Averaging seems to follow the "law of diminishing returns". As you increase the number of times the image is averaged, the improvement becomes less noticeable. At some point you should be more concerned about the light exposure of your sample.

- Frame averaging may allow a bit of "recovery" time for your fluorescent dye and could reduce the photobleaching slightly.
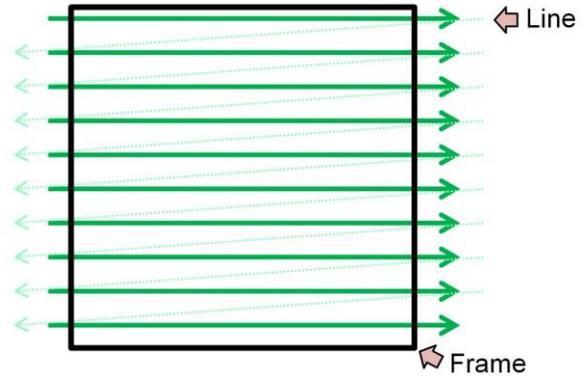
Examples of how averaging works

	Line/frame Averaging	Kalman* Averaging
A	100	100
B	92	92
C	111	111
D	94	94
	(A+B+C+D)/4=	(((((A+B)/2)+C)/2)+D)/2=
RESULT	99.25	98.75

* Kalman averaging is not an option on the FV3000 , but it is on the FV1000.

5. Pinhole size – This setting affects both the resolution of the sample (the XYZ size of each pixel/voxel in microns) and the amount of light that reaches the PMT detector.

- Ideally the pinhole should be set at (or near) 1 Airy unit. This is the best compromise between resolution and light intensity.

- If your sample is dim and noisy, one way to get more light from your sample is to open the pinhole. Opening the pinhole will reduce the optical resolution in the image, but will give you more light to work with. Opening the pinhole from 1 Airy to 2 Airy with give approximately an 8 fold increase in light. It will also reduce the XY and Z resolution by a factor of 2, or, to think of it another way, it will increase the size of your voxel 8-fold.

- Beware of doing colocalization studies with a pinhole that is much greater than 1 Airy unit. Along the same lines, colocalization studies should not be done with low NA lenses (typically low mag as well), since

the Z (axial) resolution is poor and the light from nearby (but separate) cellular compartments could appear to overlap (this would be an artifactual overlap, and not true colocalization).

6. Laser power – The laser typically starts out at 1% power (or less), and this setting can be increased if needed. The power levels can also be decreased to reduce photobleaching or photo-toxicity in live cells.

   - Increased laser power means more photons to the sample, and hopefully brighter output from the sample. There is a limit to this and eventually you can reach a point where every dye molecule is fluorescing and additional excitation will not increase the output of the sample. This high-excitation state can also speed up photo-bleaching, since it is often correlated with an increase in singlet oxygen molecules.

   - Please be aware that if you are imaging multiple colours, increasing the power to the shorter wavelength laser may cause bleaching in both the dye that you are having trouble imaging AND in the dye that is the next longest emission wavelength. This is because some dyes have long "tails" on their emission curves. Exciting a dye at a point off the peak of the excitation curve still excites the dye, if somewhat less efficiently. Example: Increasing the 488nm laser to get more GFP fluorescence may cause bleaching in the RFP signal because the RFP fluor is being exposed to two lasers (488 and 543nm). This can be a particular problem when doing Z-stacks.

- The visible light wavelength lasers on the Fv3000 are:

    - 50mW - Diode laser (405nm)

    - 50mW – Diode laser (488nm)

    - 50mW – 561nm diode laser

    - 50mW – 640 nm diode laser

- Because the lasers start out with different maximum output power levels, the appropriate starting laser power (using the sliders in the window shown on the previous page) will be different for each laser. The more powerful lasers should start at lower power levels and the weaker lasers will probably be at higher levels.

Working carefully with these options can turn a dim image into a useable one. Remember that they all have tradeoffs such as; increased light exposure in the sample (meaning that photobleaching can increase) or reduced optical resolution. Every sample is a little different, so you may need to experiment with adjusting these settings. Have fun!

FV3000 confocal - Nyquist sampling

This table assumes that the pinhole is set at 1 Airy Unit, the laser wavelength is 488nm, and the sample is being imaged under the most optimal conditions (very near to the #1.5 thickness coverslip).

Lens	NA	WD mm	Lateral (XY) resolution (um)	Axial (Z) resolution (um)	640x640		800x800		1Kx1K		2Kx2K		4Kx4K	
					Nyquist Zoom	Scan area (µm) for Nyquist sampling*	Nyquist Zoom	Scan area (µm) for Nyquist sampling*	Nyquist Zoom	Scan area (µm) for Nyquist sampling*	Nyquist Zoom	Scan area (µm) for Nyquist sampling*	Nyquist Zoom	Scan area (µm) for Nyquist sampling*
2x dry	0.08	6.2	2.914	185.2	6.8	933 x 933	5.46	1166 x 1166	4.27	1490 x 1490	2.13	2988x2988	1.07	5947 x 5947
4x dry	0.16	13	1.46	46.2	6.8	466 x 466	5.46	582x582	4.27	745x745	2.13	1493x1493	1.07	2973x2973
10x dry	0.4	3.1	0.58	7.25	6.8	186.6x186.6	5.46	233 x 233	4.27	298 x 298	2.13	597.5x597.5	1.07	1189.5x1189.5
20x oil	0.85	0.2	0.27	2.38	7.25	87.8x87.8	5.8	109.7x109.7	4.53	140.5x140.5	2.27	280.3x280.3	1.13	563x563
30x silicone oil	1.05	0.8	0.22	1.38	5.97	71.07x71.07	4.78	88.76x88.76	3.73	113.7x113.7	1.87	226.8x226.8	1.0	427x427
60x silicone oil	1.4	0.15	0.17	0.78	3.98	53.3x53.3	3.2	66.5x66.5	2.5	85.2x85.2	1.24	171x171	1.0	212x213

40x silicone lens available on request