

There are 5 things that can significantly affect the quality of images on the Zeiss 510

Doug Crome, M.S. - manager, ARL Confocal microscope facility

There are a few others (like choosing the correct filters) that have an effect, but these five probably have the most powerful affect on the confocal image.

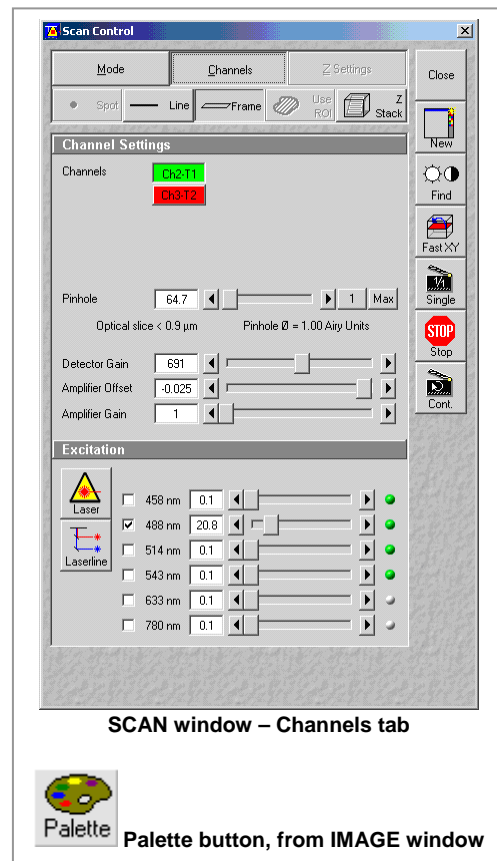
If you have a sample that initially gives a very noisy image, you may want to try aggressively adjusting some of these settings. Making these adjustments will not turn a lousy image into a perfect one, but it might give you enough information to go back to your lab and fine-tune your staining parameters. The quality of your images is significantly affected by your sample preparation.

Remember that 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, it 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 exercise in avoiding photo-toxicity and sometimes the best live-cell data has to be noisy to avoid damaging the cells.

1. **Detector gain** – The PMT detector gain setting amplifies the fluorescence signal. Higher gain values equal greater amplification. Higher gain values usually show increased noise in the image. If you can adjust some of the other settings (below) so that this value goes down, your image will usually look “cleaner” (less noisy).

- On our confocal, once you increase the gain setting to around 800 or higher, the noise level will be quite noticeable. Increasing the averaging from 2 to 4 can help with the noise, but it will not make it go away. Please be aware that increasing the averaging also doubles the cell/tissue’s exposure to light, something to consider if photobleaching is a problem.
- The cleanest looking images have PMT gain settings in the range below 500. Be aware that it may not be possible to achieve these settings with your sample.
- Make sure that you use the RANGE INDICATOR PALETTE (image window, under palette) to ensure that you do not saturate your image. RED color indicates pure white. A very tiny (occasional clusters of 4-6 pixels) amount of pure white is usually acceptable.
- Occasionally you may have some debris or 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 you sample look brighter, this is not good science.



2. **Amplifier offset** – This tells the PMT what the cutoff 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 your image.

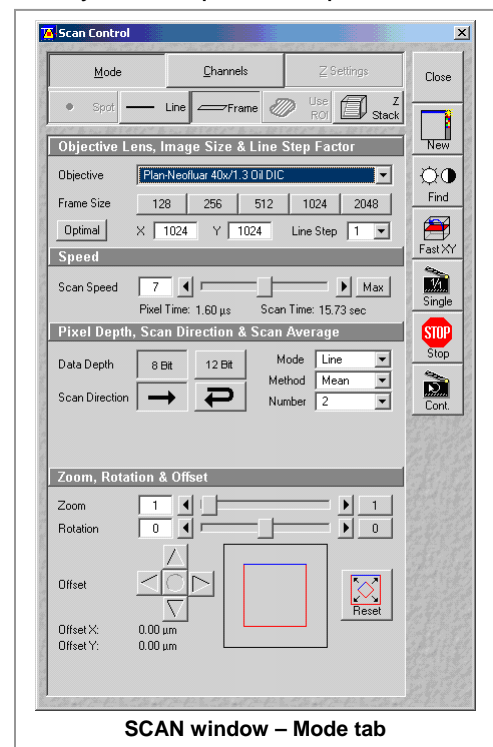
- For biological samples, this software slider should be almost all the way to the right side of the scale. It doesn't take a lot of movement on this slider to achieve the affect that you need.
- Use the RANGE INDICATOR PALETTE (image window, under palette) to check your settings. You should only have very small amounts of blue (pure black) in your image. Small amounts means a speckling of blue (like stars in the sky).
- Aggressively adjusting the amplifier offset is not the way for you to "clean up" your background. Biology has noise in it and perfectly black backgrounds do not exist in reality. If you have large areas of blue (range indicator palette) in your image, you are being too aggressive with this adjustment.
- It is also possible to adjust the amplifier offset to such an extreme that the dimmer parts of the image disappear (they become black). Do not mistake adjusting this slider with the detector gain (located right above the amplifier offset).

3. **Pinhole size** – This setting affects both the resolution of the sample (the size of each pixel 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. It can be reduced to 0.8 Airy for a very bright channel in a sample, but any smaller than this and the light intensity reaching the detector falls off very quickly.
- Because the size of an Airy unit is dependent on the wavelength of light, 1 Airy unit will give better XYZ resolution at short wavelengths (blue) and somewhat less resolution at longer wavelengths (red). If you are looking at multiple wavelengths, you need to adjust the pinholes so that the optical slice thickness (Z) is identical at all wavelengths. This may mean opening the shorter wavelength pinholes to greater than 1 Airy and closing the longer wavelength pinholes to slightly less than 1 Airy.
- 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 an 8-fold increase in light.

4. **Scan speed** – This setting affects how long the laser spends interrogating each spot within a scan. Lower numbers slow the scan down; higher numbers make it go faster.

- Slower scan speeds give improved signal to noise ratios. This will reduce the noise in the sample and allow you to reduce the detector gain.
- Slower scan speeds also increase the total light exposure of the sample, possibly leading to faster photo-bleaching.
- Scan speed affects how long it takes to acquire an image. A one number decrease in scan speed can double the acquisition time per image, something to consider if you are acquiring a Z stack of images.



- 5. Laser power** – If you are not using the laser at 100% power, this can be increased.
- Increased laser power means more photons to the sample. 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.
 - There is a rough relationship between the detector gain settings and the laser power. If you double the laser power (e.g., go from 10% power to 20%), you should be able to reduce the detector gain by the number 50 (e.g., 750 reduced to 700) and get a very similar image with reduced noise.
 - The visible light wavelength lasers on this microscope are:
 - 200mW [0.2J/s] - Argon laser (488nm and 458, 477, 514nm)
 - 5mW [0.005J/s] - Helium Neon laser (543nm)
 - 15mW [0.015 J/s] - Helium Neon laser (633nm)
 - Because the lasers start out at different power levels (see the listed milliwatts or Joules/sec for each laser), the starting percentage of laser power will be different for each. In general, the starting percentage for each laser should be approximately:
 - 10-20% power - Argon laser (488nm. *Note that the 458, 477 and 514nm lines are only 1-5% of the power available at 488nm. This means that the laser may need to begin at a much higher percent power setting to be able to image using these minor wavelengths.*)
 - 80-100% power - Helium Neon laser (543nm)
 - 60-80% power - Helium Neon laser (633nm)
 - Of the three visible light wavelength lasers, the Argon usually is the only one that can be significantly increased in output power.

Working carefully with these 5 things 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!