How to set the parameters for single point confocals

1. Scientific question

Define the scientific question for this image. Usually 1 question per image. What metrics do you want to measure? How big are the differences you want to detect? Deduct the required image resolution.

2. Objective

Choose the objective that delivers this optical resolution (0.61*l/NA). Check that the working distance, field of view and immersion medium are also sufficient.

3. Fluorophores

Choose your fluorophore combination: match fluorophore spectra with the microscope excitation and emission specifications to optimize the efficiency of imaging while avoiding bleedthrough.

4. Imaging strategy

- Imaging sequentially? Simultaneously? Combining channels? Spectrally?
- Avoid bleedthrough while optimizing the acquisition time.

5. Display

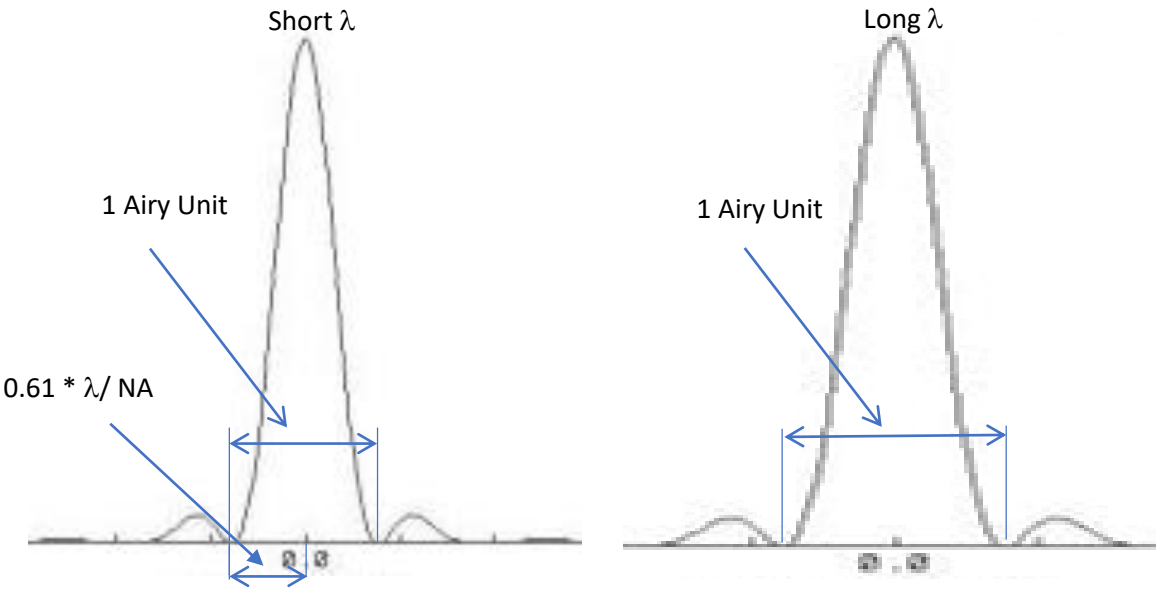
- Display the images to black and white to avoid colour bias.
- Use the LUT to push the brightness and contrast.
- Check the pixel intensity in and out of the region of interest to estimate the signal to background ratio.
- Always have the saturation and under exposure LUT on when adjusting the imaging parameters to ensure no saturation and no under exposure in or around the areas of interest.

6. Zoom/scan area

The area of interest should fill the image, minimize unnecessary information or black pixels.

7. Pixel density

- What is the pixel size that fulfills the Nyquist criterion?
  Click on Optimal/Nyquist to find out (optical resolution/2).
- Do you need to use such small pixels to answer your scientific question?
  Yes, if small distances and differences must be segmented or measured.
- Can you afford to use such small pixels?
  Only if bleaching, light toxicity, long imaging time… are not an issue.
- If undersampling, acquire and analyse undersampled images with Nyquist sampled images to estimate the impact of aliasing.



8. Pinhole size

- Should the pinhole be optimized for the shorter or longer l?

  If best contrast is required for shorter l, -> dimmer for long l.

  If best contrast is required for longer l, -> lower contrast for shorter l.

- Open the pinhole >1 AU for very dim samples that do not require high resolution in xy and high contrast.
- Close the pinhole <1 AU if your sample is very bright to get higher xy and z resolutions.

9. Laser power

- Stay low if bleaching or light toxicity are a problem (live samples, z stacks, dim samples).
- Otherwise, increase the illumination to reduce the shot noise contribution (S/N ratio).
- Long illumination with a lot of light send the fluorophores to the dark state (fluorophore depletion).

12. Detector gain and offset

- Set the gain to have no saturation at all in the areas of interest.
- High gain produces noise. Slow down the scan or increase the laser power instead.
- Turn off the laser and scan live with the underexposure mask on. Lower the detector offset until there are no 0 intensity pixels in the image.

13. Noise

- Acquire a 3 image timelapse without interval and play it to estimate the noise.
- Does the noise prevent you from answering your scientific question?
- If yes, improve the signal to noise ratio by increasing the illumination intensity, averaging or using post acquisition denoising.

14. Pixel dwell time

- If all other noise-reducing strategies are insufficient, lower the scan speed (= higher pixel dwell time). More photons reach the detector so less shot noise and better S/N ratio.
- Disadvantages: more bleaching, longer imaging, higher light toxicity, higher risk of fluorophore depletion.