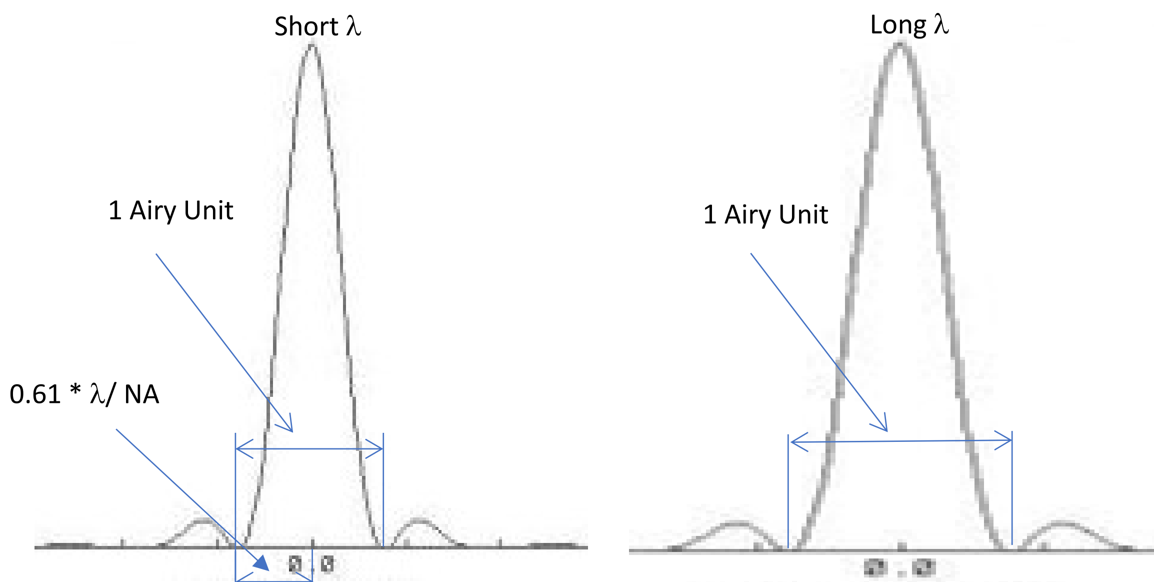
How to set the parameters for single point confocals

1. **Scientific question:** Define the scientific question for this image. Usually 1 question per image. What do you want to measure? Which resolution, working distance, field of view and immersion medium are needed?
2. **Fluorophores:** Choose your fluorophore combination: match fluorophore spectra with the microscope excitation and emission specifications.
3. **Choose an imaging strategy:**
    - Imaging sequentially? Simultaneously? Combining channels? Spectrally?
    - Avoid bleedthrough while optimizing the acquisition time.
4. **Objective:** Choose the objective that delivers the required resolution and fulfils the other criteria ($0.61*\lambda/NA$).
5. **Display:**
    - Set the images to be shown in 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.
6. **Zoom to adjust the scan area:** The area of interest should fill the image, no unnecessary information.
7. **Pixel density:**
    - What is the pixel size that fulfills the Nyquist criterion? Click on Optimal/Nyquist to find out (resolution/2).
    - Do you need to use such small pixels to reliably answer your scientific question? Yes, if small distances must be measured.
    - Can you afford to use such small pixels? Only if bleaching, light toxicity… are not an issue.
    - Beware of aliasing/Moiré artifacts for small details: Try different degrees of undersampling and compare reliably the images answer your scientific question.
8. **Pinhole size:**
    - Should the pinhole to be optimized for the shorter or longer wavelength?
        - Best contrast for short wavelength -> dimmer for long wavelength.
        - Best contrast for long wavelength -> lower contrast for short wavelength.
    - Open the pinhole >1 AU for very dim samples that do not require high contrast in xyz.
    - Close the pinhole <1 AU if very bright samples to get higher xy and especially z contrast.



9. **Laser power:**
    - Stay low if bleaching or light toxicity are a problem (live samples, z stacks, dim samples).
    - Increase to reduce shot noise contribution and improve the S/N ratio.
    - Do not saturate the fluorophores (it decreases the S/N ratio). Check by doubling laser power. The fluorescence intensity should double.
    - Do not send the fluorophores to the dark state (fluorophore depletion which decreases the S/N ratio).

10. **Detector gain and offset**
    - High gain creates noise. Set a low to medium gain and compensate by increasing the laser power.
    - Set the offset to have no pixel with a 0 intensity value in the background (using the saturation/underexposure colour mask).

11. **Noise:**
    - Acquire a timelapse of 3 images without interval/delay. What changes from one image to the next is random noise.
    - Does the random noise prevent you from answering your scientific question? If yes, improve the S/N ratio by increasing the illumination intensity, averaging or using a denoising post-acquisition tool.

12. **Pixel dwell time**
    - If all other noise-reducing strategies are insufficient, lower the scan speed (= increase the pixel dwell time). More photons go to the detector so less amplification (gain) is needed.
    - Disadvantages: more bleaching, longer imaging, higher light toxicity, higher risk of fluorophore depletion (dark state).