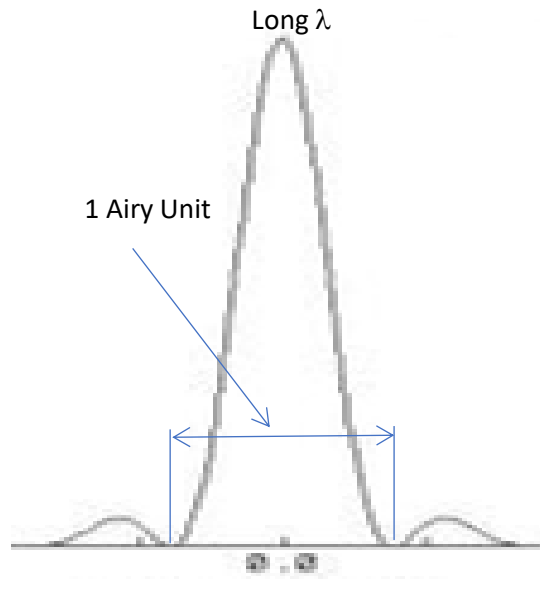
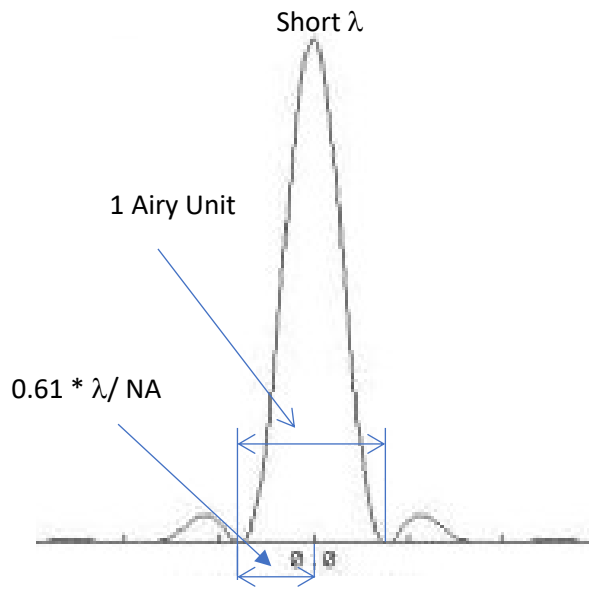


## How to set the parameters for single point confocals

1. **Scientific question:** Define the scientific question for this image. Usually 1 question per image. What do you want to measure (deduct the required resolution)?
2. **Objective:** Choose the objective that delivers the required resolution and fulfils the other criteria ( $0.61 \cdot \lambda / NA$ ). Which resolution, working distance, field of view and immersion medium are needed?
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. **Choose an 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 Lookup table on when adjusting the imaging parameters. Make sure you have no saturation, no under exposure in or around the areas of interest.
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 (optical 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.
  - If undersampling, compare with Nyquist sampled images to estimate the aliasing of undersampled details.
8. **Pinhole size:**
  - Should the pinhole to be optimized for the shorter or longer wavelength?
    - If best contrast required for shorter  $\lambda$ , -> dimmer for long wavelengths
    - If best contrast required for longer  $\lambda$ , -> lower contrast for shorter wavelengths
  - 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).