

## 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 ( $0.61 \cdot \lambda / NA$ ). 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

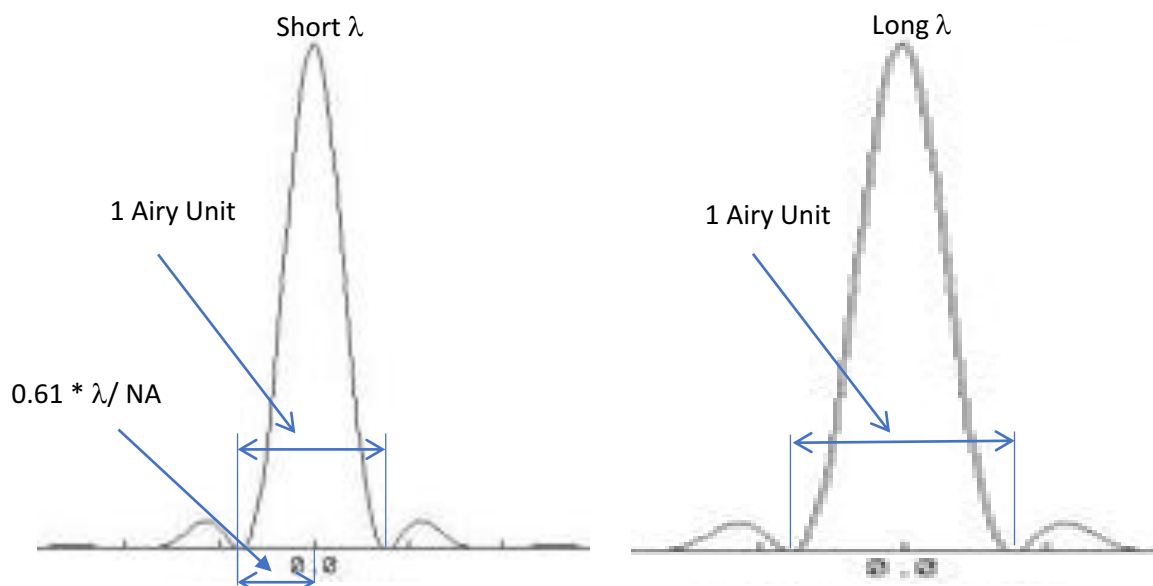
- Display the images to black and white to avoid colour bias.
- Use the LUT to push the brightness and contrast.
- Check the pixel intensity in and out of the region of interest to estimate the signal to background ratio.
- Always have the saturation and under exposure LUT on when adjusting the imaging 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 I?  
If best contrast is required for shorter I, -> dimmer for long I.  
If best contrast is required for longer I, -> lower contrast for shorter I.
- 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.