How to set the parameters for camera-based systems

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. **Objective:** Choose the objective that delivers the required resolution and fulfils the other criteria ($0.61*\lambda/NA$).
4. **Nyquist sampling:**
    - Considering the camera pixel size, calculate if the images acquired with the chosen objective fulfil the Nyquist criterium (at least 2 camera pixels should fit in the magnified resolution) or if a higher magnification (extra lens or higher magnification objective) is needed.
    - Undersampling creates artifacts. If needed, change objective or add an extra magnification lens.
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. **Saturation:** Turn on the saturation colour mask. Make sure there is absolutely no saturation in or around the areas of interest.
7. **Field of view:** Crop the camera area if the sample I doesn't fill the field of view.
8. **Field stop:**
    - Use a fully open field stop by default.
    - If the sample is bright and dense, close the excitation light field stop to just larger than the field of view. This prevents bleaching outside of the field of view and increases contrast.
9. **Binning:**
    - Use no binning by default.
    - If the full resolution of the camera is <u>not</u> needed, bin to acquire faster, get brighter images and smaller files (easier to stitch or analyse).
10. **Readout noise:**
    - Use low readout rate by default.
    - If high frame rate is needed (e.g. fast timelapse for calcium imaging), increase the readout rate. The signal to noise ratio is decreased.
11. **Illumination 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).

12. 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, choosing a slower readout rate or using a denoising post-acquisition tool.

13. Background correction:

- Subtracting the background is a must when extracting intensity information.
- You have two choices:
    i. Only remove the camera offset: Acquire an image with no illumination and a short exposure time. The average intensity over the whole image is the camera offset that must be subtracted.
    ii. Subtract the background in a region outside your sample. This background will automatically include the camera offset.

14. Use shading correction to correct for uneven illumination.