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Guidance for quantitative confocal microscopy

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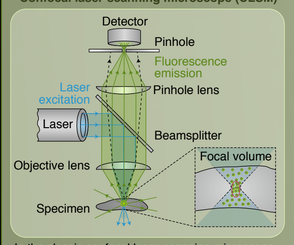
When used appropriately, a confocal fluorescence microscope is an excellent tool for making quantitative measurements in cells and tissues. The confocal microscope's ability to block out-of-focus light and thereby perform optical sectioning through a specimen allows the researcher to quantify fluorescence with very high precision. However, generating meaningful data using confocal microscopy requires careful planning and a thorough understanding of the technique. Here, we guide the researcher through all aspects of acquiring quantitative confocal microscopy images, including optimizing sample preparation for fixed and live cells, choosing the most suitable microscope for a given application, and configuring the microscope parameters. Common pitfalls such as photobleaching and crosstalk are addressed, as well as several troubling instrumentation problems that may prevent the acquisition of quantitative data. The accompanying *Nature Protocols* paper includes guidelines for analyzing and presenting confocal images and performing statistical analysis.

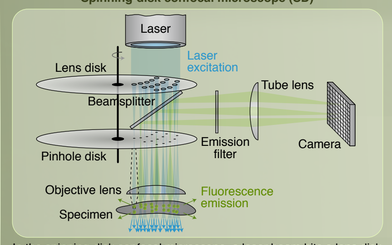
1. How confocal microscopes work

Confocal laser-scanning microscope (CLSM)



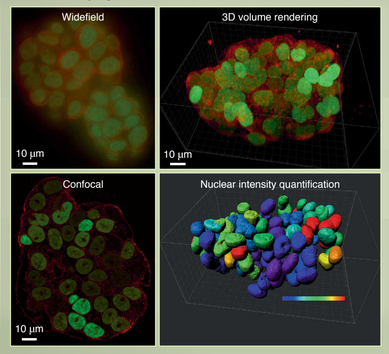
In the classic confocal laser-scanning microscope, a laser beam is focused into a specimen, where fluorescent molecules are excited throughout the entire cone of illumination (inset, green spheres). Fluorescence generated in the focal volume (red ellipse) is imaged through a pinhole onto a detector. Fluorescence from outside the focus (black dashed lines), such as from the surface of the sample, is blocked by the pinhole.

Spinning-disk confocal microscope (SD)



In the spinning-disk confocal microscope, a laser beam hits a lens disk that splits the beam into ~1000 smaller beams, which pass through a matching pinhole disk and are focused into the specimen. Fluorescent molecules (green spheres) are excited throughout the focal volume and across the field of view of the specimen as the rotating disk sweeps the pattern of laser beams. Fluorescence generated from the many focal points passes back through the corresponding pinholes and is deflected by a beamsplitter to a digital camera. Fluorescence from outside the focus (black dashed lines) is blocked by the pinhole disk.

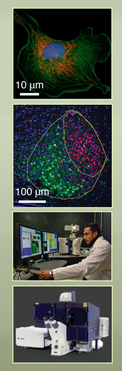
Example application: Quantifying nuclear intensities in live 3D cell culture



2. Choosing the most appropriate microscope

Confocal and alternative fluorescence microscopy techniques

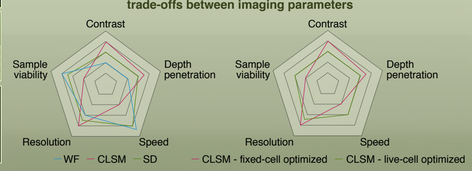
- Widefield (WF): easy, fast, and gentle for live-cell imaging, if equipped with high-quality components (lamp, objectives, filters, camera).
- CLSM: the workhorse of most facilities, with superior resolution, depth penetration, and contrast (optical sectioning strength). Optimizable for high contrast and resolution or for fast live-cell imaging.
- SD: generally optimized for live-specimen imaging with fast and sensitive (sometimes lower-resolution) cameras.
- Multiphoton (MP): more than twice the depth penetration of a CLSM, with excitation limited to the focal plane. Lower resolution with near-infrared light.
- Total internal reflection fluorescence microscopy (TIRF): extremely thin optical slice (~100 to 200 nm), but excitation occurs only adjacent to the coverslip (no depth penetration).
- Super-resolution: lateral resolutions may reach 120–160 nm (Airyscan, Lightning),100–140 nm (structured illumination microscopy (SIM)), 25–50 nm (stimulated emission depletion microscopy (STED)), 20-40 nm (stochastic optical reconstruction microscopy (STORM)), or even 2–5 nm (MINFLUX).
- Light-sheet microscopy: Fast optical sectioning of larger specimens such as zebrafish embryos or cleared tissues and organs.



Which parameters are important for answering your biological question?

- Sample viability: Use a sensitive microscope to reduce illumination power, particularly for live-cell imaging.
- Speed: For live-cell imaging, match the acquisition speed to the dynamics that are being studied.
- Resolution: Consider the size of the features that must be resolved, both laterally and axially.
- Contrast: Confocal and related optical sectioning techniques improve contrast by removing out-of-focus fluorescence.
- Depth penetration: Confocal can generally image only 50–100 µm deep because of absorption and scattering in biological specimens.

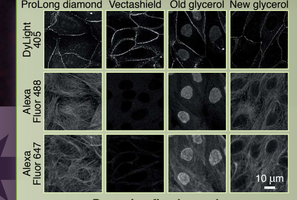
Radar plots help to visualize the trade-offs between imaging parameters



3. Planning your experiment

- Carefully consider what positive and negative control samples will be required.
- Run a small pilot project from sample prep through to analysis. This will give you the chance to adapt image acquisition to ensure the data are suitable for answering your biological question.
- Your eyes will be drawn to capture fields of view that suit your hypothesis. Remove bias by running blinded studies: have a colleague label your slides for you in code, and only reveal the control and experimental conditions when the analysis is finished.
- For tissue sections, rather than selecting small fields of view on a confocal microscope, consider thin sectioning (10 to 15 µm) together with whole-slide scanning and whole-slide analysis.

4. Hazards of sample preparation



Mounting medium

- Appropriate antifades can reduce photobleaching significantly.
- Vectashield works for many dyes, but it quenches some (e.g., Alexa Fluor 647). Check or test compatibility.
- Old reagents and mountants can produce strange results. (Compare old and new glycerol – there should be no nuclear labeling here.)
- Avoid mountants that contain DAPI.
- Hardening mountants may flatten 3D structures.
- Seal the slide when using non-hardening mountants.
- The refractive indices of hardening mountants change over time as they cure.

Preparing fixed samples

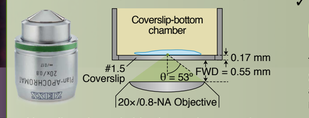
- Optimize fixation, permeabilization, labeling and mounting.
- For the best imaging quality, use #1.5 coverslip thickness and grow cells directly on the coverslip.
- Use bright, stable fluorophores. Some flow cytometry dyes are not suitable for microscopy (e.g., fluorescein, phycoerythrin).
- Test antibodies, optimize concentrations, and use controls.

Preparing live cells

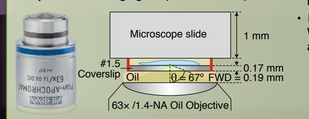
- Avoid fluorescent proteins that aggregate or mis-localize.
- Vital dyes that label particular compartments (CellTracker, Hoechst) can be toxic to cells over time – keep concentrations low.
- Use #1.5 coverslip-bottom dishes and chambers for high resolution and high sensitivity.
- Use microscope incubators to control temperature, CO_2, and humidity during imaging.

5. Objective lenses

Use high-NA lenses for:
✓ Better resolution: 2× NA = 2× resolution
✓ Greater sensitivity: 2× NA = 4× sensitivity



- Use oil or glycerol objectives to increase the NA for fixed samples.
- Use water, glycerol or silicone oil immersion objectives for imaging deep into live samples.

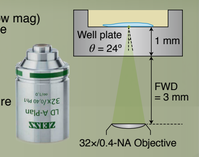


FWD, Free working distance

Use low-NA lenses for:
✓ Large field of view (low mag)
✓ Long working distance
✓ Large depth of field

$NA = n \cdot \sin\theta$

The numerical aperture (NA) of a lens is just as important as the magnification.



- For CLSMs, a lower-magnification objective provides a larger field of view (FOV). Choose an objective whose NA provides the required resolution, and adjust the number of pixels to match the FOV.
- Many SDs are optimized for 60× or 100× objectives. While SDs are ideal for live-cell imaging, fixed cameras and pinhole disks offer less flexibility.

n = refractive index of immersion medium

$n_{air} = 1.00$
$n_{water} = 1.33$
$n_{silicone} = 1.40$
$n_{glycerol} = 1.47$
$n_{oil} = 1.52$

6. Resolution and sampling

Rayleigh criterion: a rule of thumb for resolution

Lateral resolution depends on the NA of the objective and the wavelength (λ) of light.

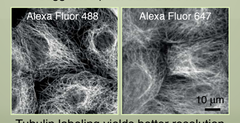
$$r_{xy} = \frac{1.22\lambda}{2NA}$$

Nyquist sampling

Pixels should be 2 to 3 times smaller than the resolution limit or the smallest feature that needs to be resolved.

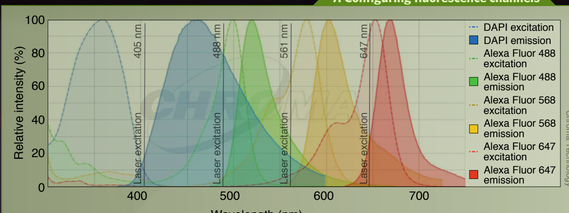
Resolution example

$\lambda \approx 500$ nm (eGFP fluorescence)
NA = 1.4 (oil immersion objective)
Expected resolution: $r_{xy} \approx 200$ nm
Suggested pixel size: ≤100 nm

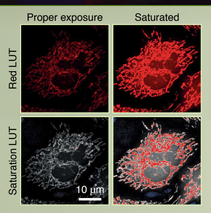


Tubulin labeling yields better resolution with shorter wavelength.

7. Configuring fluorescence channels

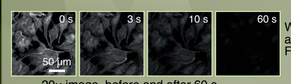


- With sequential acquisition, 4 channels can typically be separated without cross-talk (see screenshot from Chroma's Spectra Viewer, above).
- Built-in wizards usually do not produce the optimum configuration.
- Simultaneous imaging of fluorescence channels should be reserved for fast imaging of dynamics, not fixed samples.
- Semi-sequential imaging works only if fluorophores are the same intensity (usually DAPI is too strong and will still bleed through to the red channel).
- Line-switching sequential acquisition is best to avoid cross-talk.

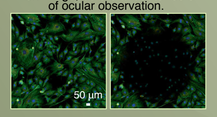


- CLSM detectors have poor dynamic range. For fixed samples, increase the gain, laser power, or pixel dwell time to use the full dynamic range.
- Special look-up tables (LUTs) can help assess whether there are saturated pixels (above) or an inappropriate offset (zero-intensity pixels).
- Changes in intensity are easier to visualize in grayscale images compared to color (avoid red and blue).

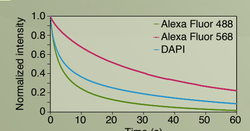
8. Finding the field of view and photobleaching



20× image, before and after 60 s of ocular observation.



With the epi-fluorescence lamp on full power at 63×, even the stable fluorophore Alexa Fluor 488 can lose half its intensity in 3 s.

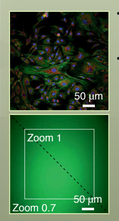


Minimize photobleaching and phototoxicity while finding your field of view in the binoculars

- Turn the lamp down to its lowest setting, usually 10% (even lower for live cells). Dim the room lights and allow your eyes to adjust to the darkness before viewing the specimen.
- Use the fluorescence shutter liberally! If you turn away from the microscope for even a second, close the shutter.
- For very sensitive experiments, use transmitted light (e.g., differential interference contrast (DIC)) microscopy to find your cells.

9. Troubleshooting instrumentation issues

Non-uniform illumination

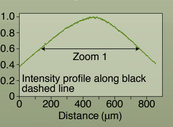


The problem

- This 20×/1.0 NA intravital imaging objective is particularly non-uniform, varying by 40% (corner to center) at zoom 1.
- Most objectives on a CLSM have a non-uniformity of at least 10%.

Potential solutions

- Restrict imaging to a smaller region in the center of the field of view, using zoom 2 or higher.
- Use uniform slides to measure the illumination, and apply 'flat-field' or 'shading' correction.



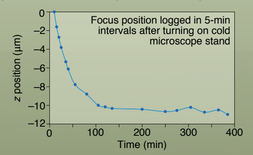
Focus drift

The problem

- The focus on most microscope stands can take hours to stabilize, even without incubators.
- Focus changes affect intensity for confocal images.

Potential solutions

- For live-cell imaging, use hardware autofocus devices.
- For longer fixed-cell imaging (super-resolution, tiling), plan for 3-h warm up or leave the microscope stand turned on 24/7.
- Try to ensure room temperature stability.



Laser instability and power variation

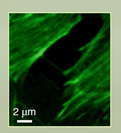
The problem

- Laser fluctuations >10% are not uncommon, even after 1-h warmup.
- When a laser is replaced or the instrument is serviced, the power changes are rarely measured and recorded.
- Laser powers (same λ) vary 50-fold across confocal microscopes.

Potential solutions

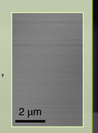
- Users should warm up the system for 1 h before imaging and repeat experiments in a different order.
- Facility staff should use power meters to track fluctuations and long-term changes in power.

Jitter and stripes



The problem

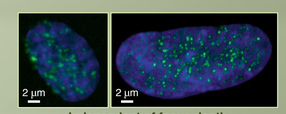
Jitter or stripes appear in the image. They may not be obvious when imaging some samples (e.g., with punctate labeling) but they affect the data nonetheless.



Potential solutions

- Use a slide with linear structures such as actin filaments (left) to detect jitter and a uniformly fluorescent slide (right) to detect stripes.
- Vibrations are the most obvious cause. Check that the optical table is floating properly; remove components with fans from the table. Don't bump the table.
- Check if bi-directional scanning is on. Recalibrate it or turn it off.
- Check for a loose microscope component (stage, objective lens) and tighten.
- Room lights affect some modes of imaging (e.g., multiphoton with non-descanned detectors).
- Test for hardware problems: e.g., fluctuations in the laser or noisy acousto-optic tunable filter.
- Check that the sample is properly fixed.

10. Troubleshooting blurry images



Independent of focus depth

- Is the objective lens dirty (e.g., dried oil on air objective) or damaged?
- Is the objective pushing up against the stage insert? The spring-loaded front element may be compressed.
- Was the right immersion medium used? Check for air bubbles.
- Is the sample itself old or poorly prepared? Fluorophores may diffuse from the structures they were labeling.

Focused >10 µm beyond the coverslip

- Check the coverslip thickness (usually #1.5 is required).
- Does the objective lens have a correction collar, and is it properly adjusted?
- Are you using a high-NA water objective with fixed cells (in glycerol-based mountant) or a high-NA oil objective with live cells? Refractive-index matching is crucial >10 µm beyond the coverslip.

References

For more details, including guidelines for quantitative image analysis and statistics, refer to the full paper: Jonkman, J., Brown, C.M., Wright, G.D., Anderson, K.I., & North, A.J. Tutorial: guidance for quantitative confocal microscopy.

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