



Seeing beyond

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 troubleshooting 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. New 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 (green spheres). Fluorescence generated in the focal volume (red ellipse) is imaged through a pinhole onto a detector. Fluorescence from outside the focus, such as from the surface of the sample, is blocked by the pinhole (black dashed lines).

#### Spinning-disk confocal microscope (SD)

In the spinning-disk confocal microscope, a laser beam hits a lens 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 detected by a beam splitter to a digital camera. Fluorescence from outside the focus (black dashed lines) is blocked by the pinhole disk.

### 2. Choosing the most appropriate microscope

#### Confocal and alternative fluorescence microscopy techniques

- Widefields (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). Optimize 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 coupling into depth penetration
- Super-resolution, lateral resolutions may reach 100–160 nm (Alyssa's 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 2–4 nm (MINI-LUX)
- 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 stability: Use a sensitive microscope to reduce illumination, 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 nm deep because of absorption and scattering in biological specimens

### 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 nm) together with whole-slide scanning and whole-slide analysis.

### 4. Hazards of sample preparation

#### Mounting medium

- Appropriate antifades can reduce photobleaching significantly
- Wet-mount media: for many dyes, but it quenches some (e.g. Alexa Fluor 647). Check for red camera compatibility
- Old organic and inorganic can produce strange results. (Compare old and new glycerol – there should be no nucleus labeling here)
- Avalon mounts can contain DAPI
- Hardening mounts may label 3D structures
- Seal the slide: increasing non-hardening mounts will
- The refractive indices of hardened mounts change over time as they cure

#### Preparing fixed cells

- Avoid fluorescent proteins that aggregate or fluoresce and mounting
- Use bright, stable fluorophores
- Some low cytotoxicity dyes are not suitable for microscopy (e.g. fluorescein, phycoerythrin)
- Test antibodies, optimize concentrations, and use controls.

#### Preparing live cells

- Optimize fixation, permeabilization, labeling and mounting
- For the best imaging quality, use #1.5 coverslip thickness and grew cells directly on the coverslip
- Use bright, stable fluorophores
- Test antibodies, optimize concentrations, and use controls.

### 5. Objective lenses

#### Use high-NA lenses for:

- ✓ Larger field of view (low mag)
- ✓ Greater sensitivity: 2x NA = 4x sensitivity

#### Use low-NA lenses for:

- ✓ Larger field of view (low mag)
- ✓ Long working distance
- ✓ Larger depth of field

NA = n × sin θ

The numerical aperture (NA) of a lens is just as important as its 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 60x or 100x objectives. While SDs are ideal for live-cell imaging, fixed cameras and pinhole disks offer less flexibility.

$n$  = refractive index of immersion medium  
 $\theta$  = half-angle of light at NA

### 6. Resolution and sampling

#### Rayleigh criterion: a rule of thumb for resolution

Lateral resolution depends on the NA of the objective and the wavelength ( $\lambda$ ) of light.

$$\Delta r_{\text{Rayleigh}} = \frac{1.22 \lambda}{2NA}$$

#### 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

$\lambda = 500$  nm (eGFP fluorescence)  
 NA = 1.4 (oil immersion objective)  
 Expected resolution:  $\Delta r = 200$  nm  
 Suggested pixel size:  $\leq 100$  nm

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).

### 7. Configuring fluorescence channels

- DAPI emission
- Alexa Fluor 488 emission
- Alexa Fluor 488 excitation
- Alexa Fluor 488 emission
- Alexa Fluor 568 emission
- Alexa Fluor 647 excitation
- Alexa Fluor 647 emission

- 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 dynamic, 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.

### 8. Finding the field of view and photobleaching

With the right fluorescence lamp and filter choice at 800, even the stable fluorochrome Alexa Fluor 488 can lose half its intensity in 3 s.

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

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

- 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

#### Laser instability and power variation

The problem: Laser fluctuations >10% are not uncommon, even after 1-h warm-up.

When a laser is replaced or the instrument is serviced, the power changes are rarely measured and recorded.

Laser powers (some 2) vary 50-fold across confocal microscopes.

#### 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.

#### Independent of focus depth

Is the objective lens dirty (e.g., dust or oil on an objective) or misaligned?

Is a 0.5 objective panning up, gaining the stage height? The spring-loaded front element may be compressed.

Was the right immersion medium used? Check for air bubbles.

Is the sample fixed or properly prepared? Fluorophores may diffuse from the structures they were labeling.

Focused  $\leq 10 \mu\text{m}$  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 mounts) or a high NA oil objective with live cells? Refractive-index matching is crucial  $>10 \mu\text{m}$  beyond the coverslip.

### 10. Troubleshooting blurry images

#### Non-uniform illumination

The problem: The focus on most microscopes stands can take hours to stabilize, even without incubators.

The focus changes affect intensity for confocal images.

#### Potential solutions:

- For live-cell imaging, use hardware autofocus devices
- For longer live-cell imaging (super-resolution, TIRF), plan for 3-h warm-up to leave the microscope stand warmed on 24/7
- Use uniform slides to measure the illumination, and apply flat-field or shading correction.

#### Focus drift

The problem: The focus on most microscopes stands can take hours to stabilize, even without incubators.

The focus changes affect intensity for confocal images.

#### Potential solutions:

- For live-cell imaging, use hardware autofocus devices
- For longer live-cell imaging (super-resolution, TIRF), plan for 3-h warm-up to leave the microscope stand warmed on 24/7
- Use uniform slides to measure the illumination, and apply flat-field or shading correction.

### 11. Troubleshooting blurry images

#### Confocal microscope laser fluctuations

488-nm laser powers (mW) by instrument:

- Zeiss LSM1010: 4.8
- Zeiss LSM710: 4.8
- Zeiss LSM700: 4.8
- Nikon A1R10: 4.8
- Olympus FV1000: 4.8
- Leica SP8: 4.8
- Leica SP5: 4.8
- Leica SP2: 4.8
- Leica SP1: 4.8
- Leica SP10: 4.8

### 12. Troubleshooting blurry images

#### 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. *Nat. Protoc.* <https://www.nature.com/articles/nprot20200313> (2020).

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### 13. Troubleshooting blurry images

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