

# The Quagmire of Quantitative Confocal Microscopy – a Tutorial

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## Key references for associated links

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## Abstract

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 on a confocal requires careful planning and a thorough understanding of the technique. In this tutorial, the researcher is guided 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. Suggestions are offered for planning unbiased and rigorous confocal microscope experiments. A potential quagmire of less obvious problems that may prevent the acquisition of quantitative data is revealed, and options for navigating the quagmire are proposed. Finally, guidelines for analyzing and presenting confocal images in a way that maintains the quantitative nature of the data are presented, and statistical analysis is discussed.

Keywords: confocal, microscopy, quantitative, fluorescence



## Introduction

Confocal microscopes have a modest advantage over regular “widefield” (epifluorescence) microscopes in resolution, but the main advantage is their unique ability to perform optical sectioning. In a widefield microscope, the acquired images are a superposition of sharp features from the focal plane and blurry features from out-of-focus; but a confocal microscope blocks the latter, resulting in a sharp, high contrast image from the focal plane alone (Figures 1a and 1b). With a high-resolution objective lens, a confocal microscope can generate optical sections thinner than 1  $\mu\text{m}$  without having to physically slice the sample. It can therefore be used to quantify the intensities and investigate the spatial arrangement of fluorescent molecules with very high precision, useful for assigning the localization of molecules to specific cellular compartments or assessing the colocalization of different molecules. A single confocal image (or “slice”) may be sufficient for quantification if it is representative of the entire thickness of the sample, but one can also take a series of confocal images while changing the focus to produce a 3D dataset (or “z-stack”), enabling the reconstruction and quantification of the entire volume (Figures 1c and 1d).

The essential component common to all confocals is one or more strategically placed pinhole apertures. Figure 2a shows a schematic of the classic confocal laser-scanning microscope (CLSM). A laser beam is focused into a specimen, where it generates fluorescence throughout the entire cone of illumination. Fluorescence that comes from the focus is collected by the objective lens and focused by a second lens through a carefully aligned pinhole onto a detector. The name “confocal” derives from the position of the pinhole(s) in the microscope’s lightpath (in a CONjugate FOCAL plane with the sample), where it blocks any fluorescence that did not originate from the focus. As the focused laser only excites fluorescence in a tiny spot in the specimen, the CLSM uses scanning mirrors to sweep the beam across the specimen, generating an image pixel-by-pixel. Since you typically have to dwell for about 1  $\mu\text{s}$  on each pixel, it takes about 1 second to generate a modest 1024 x 1024 pixel image. To capture fast dynamics in live specimens, small regions of interest can be scanned or a different confocal geometry might be needed. A spinning disk (SD) confocal microscope illuminates the sample using an array of pinholes, arranged in a special pattern on a disk, to create hundreds of focused beams. The fluorescence is then collected back through the pinholes (creating the optical section) and detected using a digital camera – effectively parallelising multiple confocal lightpaths (Figure 2b). However, since SDs are usually optimized for speed at the expense of resolution and flexibility, they are often less well suited for fixed specimens. The relative advantages and disadvantages of different confocal modalities are discussed below.

Despite being quite sophisticated, it is relatively straightforward to snap pictures on a modern confocal microscope. However, generating **quantitative data** on a confocal requires thoughtful planning and careful execution at all stages of the experiment. Some applications have a clear requirement for quantification, such as measuring a change in expression levels of a fluorescently labeled protein. In other cases, the reason for requiring quantification may be less obvious. For example, it is tempting to assume that preliminary experiments will not require quantification, yet quantitative images are necessary even for reliable qualitative comparisons (“sample A is brighter than sample B”). Quantitative images are also necessary to apply a consistent intensity threshold during cell counting and segmentation. Ultimately, most experiments that use confocal microscopy require (or benefit from) quantitative acquisition to produce meaningful data.

Quantitative confocal microscopy involves rigorous specimen preparation, careful selection of an appropriate microscope for a given application, and stringent microscope set up and operation in a way that enables equal and fair assessment of control and experimental conditions. Care must be taken to avoid bias and ensure that differences are statistically meaningful. All subsequent image

processing and analysis steps must be performed cautiously and with integrity. Sadly, even the latest confocal microscopes suffer from common but less obvious problems that can dramatically affect their ability to generate measurable data<sup>1</sup>. Illumination intensity may vary substantially from the center of the image to the periphery (often as high as 40%). Stage drift often plagues time-lapse imaging, where a perceived change in intensity is in fact a change in focus (0.5  $\mu\text{m}$  change in focus over 5 min is typical). Particularly troubling are laser power fluctuations that can alter the intensity by 10% to 25% over a typical 3-hour imaging session! This quagmire of unexpected issues must currently be navigated by careful quality control on the part of the user, though future hardware improvements by manufacturers could also be greatly beneficial towards enabling reliable quantitative confocal microscopy.

## Preparing samples for quantitative fluorescence microscopy

Sample preparation is one of the most critical steps in any fluorescence microscopy experiment, leading to the microscopists' motto of "Garbage in = garbage out". The higher the performance of the microscope, the more likely it is to reveal inadequate sample preparation. All microscopy work benefits from optimising the lightpath between the objective lens and the focal plane in the sample. A particularly insidious problem is spherical aberration, which leads to image blur, particularly in the z-axis, and loss of signal intensity. Spherical aberrations result primarily from the use of inappropriate coverslips and refractive index (RI) mismatch between the microscope objective immersion medium and the sample (more details in "Setting up the microscope"). Modern objective lenses are designed to be used with 170  $\mu\text{m}$  thick coverslips (#1.5), optimally high precision versions (#1.5H) with only  $\pm 5$   $\mu\text{m}$  variance. For high-resolution imaging, any other coverslip thickness requires use of an objective with an adjustable correction collar. It is best to grow cultured cells directly on the coverslip to minimize the distance between the coverslip and the specimen. Multi-well microscope slides with removable gaskets can be problematic if there is any kind of "spacer" or residual substance left on the slide after removing the gasket. Dishes or chambers with a #1.5 glass coverslip bottom are very useful when working with live cells on an inverted microscope (see below).

## Preparing fixed cells and tissues

For fixed samples, the most critical sample preparation steps are fixation, permeabilization, labelling and mounting. Additionally, tissues require sectioning at an appropriate thickness. For widefield microscopy, sections are generally 4 to 10  $\mu\text{m}$  thick, but for confocal microscopy 10 to 40  $\mu\text{m}$  is more common. Antibody and fluorophore penetration beyond 40  $\mu\text{m}$  is challenging and may require modified labeling protocols or perfusion of probes. Tissue clearing techniques can be used to render the tissue more transparent for thicker sections or even whole organs<sup>2</sup>.

### Fixation

There is no such thing as a "standard" fixation protocol: fixation must be optimized for every cell or tissue type. Fixation methods can be chemical or physical. In biomedical research, chemical fixations, typically using cross-linking approaches (aldehyde fixation) or precipitation methods (methanol, ethanol, acetone) are most commonly used<sup>3</sup>. Physical methods such as high pressure freezing, more common in electron microscopy, offer superior preservation of some samples, including worms and yeast<sup>4</sup>. In chemical fixation, the trick is to address what is known as the immunocytochemical compromise – sufficient cross-linking is necessary to preserve morphological details, yet too many cross-links can reduce antigenicity and prevent antibody penetration. Hence fixation must be optimized for each target structure and antibody. Very stable tissue structures (e.g. cell-cell junctions) are easily retained in place, such that gentle methanol fixation may suffice for the purposes of identification<sup>5</sup>, while other tissue components, such as soluble proteins, require more stringent fixation. Moreover, polyclonal antibody staining is generally more resistant to chemical fixation than staining with monoclonal antibodies, since they detect multiple epitopes<sup>6</sup>. Glutaraldehyde (GA) is a bivalent cross-linker, while paraformaldehyde (PFA) is monovalent. This renders GA more useful than PFA alone for certain cellular structures such as microtubules (use low GA percentages to minimize loss of antigenicity). Be aware of the different sources of formaldehyde (FA): formalin solution, commonly used by pathologists, also contains methanol, hence FA prepared in pure solution from PFA is generally superior to diluted formalin for immunocytochemistry. The duration and temperature of fixation must also be optimized. For antibodies or structures that do not withstand aldehyde fixation well, try ice cold methanol, acetone or ethanol. Conversely the fixation of cytoskeletal structures can often benefit from warming the fixative, particularly for

mammalian cells, to prevent a cold shock to the cells. New, improved tissue preservation methods are constantly being developed<sup>7,8</sup> and should be tested.

### *Permeabilization*

Antibodies and fluorophores are generally unable to penetrate the plasma membrane and reach the cytoplasm, unless detergents are used to make holes in the membranes. The permeabilization regime and detergent choice must be optimized. Some structures are better revealed by a brief permeabilization step prior to fixation, but soluble proteins will be removed, so most protocols either fix first, or fix and permeabilize simultaneously. Try combinations of multiple steps and compare the results. Insufficient permeabilization of tissue sections can lead to poor antibody penetration only into the top and/or bottom of the tissue, while too much permeabilization can lead to loss or artifactual redistribution of the target<sup>9</sup>. Triton-X is a harsher detergent than saponin, which interacts selectively with membrane cholesterol, producing small holes in the plasma membrane without affecting cholesterol-poor membranes in mitochondria and the nuclear envelope<sup>10</sup>. Saponin's effects are reversible, so it must be included in all solutions during the labelling procedure. Finally, when labelling surface proteins, note that the small amount of methanol in FA from formalin can cause some cell permeabilization and allow labelling also of internal structures.

### *Labelling*

The next step is to select an appropriate labelling method to specifically target the structure of interest. This could involve antibody staining, or a direct labelling approach (the latter may negate the need for permeabilization). Genetic approaches – fluorescent proteins (FPs), or tags such as SNAP and HALO tags<sup>11</sup> - have been increasingly used over the past decades, but antibody approaches are still prevalent and useful for verifying the localization of genetically tagged proteins to the correct cellular structures. The use of nanobodies to couple more photostable organic dyes to genetically expressed FPs is particularly useful for super-resolution microscopy techniques<sup>12</sup> but can also be applied to confocal microscopy. Companies also sell reagents for tagging specific cellular structures, such as DNA dyes for the nucleus, or fluorescent markers for specific organelles (membranes, lysosomes, mitochondria, etc.).

There is no such thing as a “standard” immunolabelling protocol for cells or tissues: entire books have been written on this topic alone<sup>6</sup>. Blocking steps (with bovine serum albumin or serum for example) should be introduced when required (inevitably for tissues, often for cell monolayers), to minimize non-specific binding of primary or secondary antibodies (also a source of fluorescence background) or to block endogenous enzyme activity when using enzymatic labelling approaches. Appropriate antibody concentrations must be established, plus the optimal duration and temperature of labelling. Although counterintuitive, reducing antibody concentrations often leads to better staining as specific binding is retained at the expense of non-specific binding. The optimal duration can vary widely from 20-30 mins for cultured cells to hours or even days for tissue sections or cleared organs. If labelling at room temperature results in a high level of background signal, incubation of the sample with primary antibody overnight at 4°C may increase specificity. Incubation times with secondary antibodies (typically one hour) should be reduced to lower nonspecific background. Sufficient washing steps are also critical, with many, shorter washes being superior to a couple of long ones. Also note that total labelling and washing times for weakly fixed tissue or cells should be minimized to prevent loss of cellular components from the sample. Finally, a variety of controls are essential for both primary and secondary antibodies including minus primary antibody and isotype controls<sup>6</sup>. Positive controls are often as important as negative controls, when the quality of the primary antibody is unknown<sup>13,14</sup>. The ideal negative control for primary antibody staining is generally knock down of the protein of interest to show loss of all antibody staining.

Use the brightest and most stable fluorophores available. Microscopists must avoid many common flow cytometry dyes, such as fluorescein and phycoerythrin (PE), that are prone to rapid photobleaching. Fluorescent tags designed specifically for microscopy are generally superior, such as the Alexa Fluor, Cyanine, DyLight, and Silicone Rhodamine dyes, and the non-commercial Janelia Fluor dyes. Exercise careful choice in the wavelength of chosen fluorophores. Several companies (such as Chroma, Semrock, Omega and Thermofisher) offer online spectra viewers that display excitation and emission spectra of fluorophores, helping match to the available lasers/filters on the microscope and minimize overlap between multiple probes. If signal intensities of different fluorophores are balanced, most confocal microscopes can easily image four fluorophores without significant cross-talk. Modern systems capable of spectral imaging tempt users to label with five or more fluorophores at once. However, this complicates the sample preparation, imaging and analysis, so consider whether all five components need to be labelled at the same time, or whether the same information can be obtained by labelling two successive samples. When tissue autofluorescence is a major problem, avoid shorter wavelength (blue/green-emitting) dyes. However, for high resolution work, long wavelengths (e.g. near infrared) will reduce the achievable resolution (Figure 3). Microscope-induced chromatic aberrations, where different wavelengths become focused to different z-positions, are most problematic when combining blue-emitting dyes with other colors<sup>15</sup>. Certain objective lens types (e.g. Plan Apochromat) are better corrected than others, but very few are well corrected across the entire spectrum. Chromatic aberration will critically affect any colocalization analysis, and should be measured and corrected for using multicolour microspheres<sup>16</sup>.

### *Mounting*

Sometimes researchers unwittingly ruin their carefully prepared specimens at the final hurdle of mounting the sample. Inappropriate mountants cause problems in two major areas – loss of 3D information and loss of signal. Glycerol-based or uncured mountants will preserve 3D information (critical for colocalization analysis) better than hardening mountants, which squash the sample flat. Mountants designed to give a certain refractive index after hardening (such as Prolong Diamond) can be prevented from curing to preserve 3D structure by immediately sealing around the entire coverslip edges using a quick-drying nail polish (colored quick-dry types are the best, as it's difficult to spot gaps with clear polish). It is important to know whether the antifade reagent included in the mountant is compatible with the fluorophores used. No one anti-fade works with all fluorophores, and an inappropriate anti-fade may drastically reduce signal intensity (Figure 4) as well as proving ineffective against photobleaching. For example, Vectashield, which preserves fluorescein optimally and is compatible with many other blue/green dyes, markedly reduces fluorescence from some far red dyes including Alexa Fluor 647<sup>17</sup>. Published information on the incompatibility of specific antifade reagents with different fluorophores is scarce. Thus, test different mountants on your chosen dyes and compare the results empirically. Sometimes completely inexplicable results will be obtained (Figure 4) which would otherwise have been attributed to poor antibody labelling. Critically, never use a mountant that contains DAPI as a nuclear counterstain, as this increases background fluorescence. Instead, perform DNA staining as a separate step before mounting. Be aware that DAPI staining can also photo-convert to give green fluorescence with extended UV excitation<sup>18</sup>.

### *Quality control – fixed samples*

Before using the confocal microscope, check to see if the sample preparation was successful for all fixed samples on a widefield fluorescence microscope. Check that negative controls lack fluorescence and the staining of positive controls is bright and specific. A confocal microscope cannot remove non-specific staining, nor will it increase signal brightness. In fact, signal intensity often appears weaker on the confocal microscope since emitted light is acquired only from a thin optical section, rather than the entire specimen thickness. However, the background and autofluorescence are

typically reduced with a confocal microscope, thanks to optical sectioning and to the ability to select narrow band emission filters or tune the spectral detection. Finally, check samples for antibody precipitates and for strange cell morphology that could indicate poor fixation or unhealthy cells (e.g. rounding up or blebbing) – a transmitted light (brightfield/phase contrast/DIC) image can help with this assessment. If the sample is suboptimal, start again.

### Preparing live cells

Live cell work avoids many of the concerns of fixed sample preparation listed above, but introduces its own challenges: most notably, how to label structures or molecules of interest and image them without compromising viability or disrupting the complex machinery of the cell<sup>19,20</sup>.

### Fluorescent protein labeling

With an overwhelming number of fluorescent protein (FP) variants now available (<https://www.fpbases.org/>), one must carefully select the most appropriate ones according to the microscope's specifications (e.g. lasers and filters), the approach being used (e.g. time-lapse imaging, multi-colour, photoactivation, etc.), and the protein, cellular compartment or even the specific organism being studied<sup>21,22</sup>. While the latest super-bright variants may appear tempting, the problem of fluorescent protein aggregation<sup>23</sup> or mislocalization may render the tried and tested older FPs, such as eGFP, the best choice for many studies. Ongoing searches of the most recent literature are essential for appropriate probe selection. Fluorescent proteins can be introduced into cells using transient transfections with lipid-based reagents (e.g. lipofectamin, effectine), electroporation, or viral vectors. Monitor fluorescent protein levels and adjust experimental conditions or plasmids (e.g. use less efficient promoters) to reduce the possibility of overexpression artifacts. Cells can be left in selection media for an extended period of time (2-3 weeks) to develop stable cell lines and sorted to isolate cells with appropriate levels of expression. FPs are not very bright or photostable so genetic tags such as SNAP and HALO, which enable the use of optimized fluorescent dyes, are particularly suitable for light intensive applications such as super-resolution approaches<sup>11,24,25</sup>.

### Vital dye labeling

There is an ever-growing arsenal of vital dyes available for labeling entire cells (e.g. for tracking) or organelles. Most dyes can simply be added to cells from a stock solution and incubated for 5-10 minutes. Adding any dyes to live cells will inevitably cause perturbations to the cellular physiology and interfere with normal function so dye concentrations must be minimized. When labelling DNA for long-term imaging experiments, avoid dyes that intercalate into the DNA. Either FP-labelled histones, or SiR-Hoechst, can be used without noticeably affecting cell division for some cell types. Many small molecule dyes such as MitoTrackers<sup>TM</sup> can be phototoxic so they should be avoided or used at minimal concentrations for longer experiments. In our experience, most dyes can be diluted hundreds of times lower than suggested in manufacturer protocols. Minimize cell stress by finding the lowest dye concentration that still provides adequate visualization of the structures of interest.

### Live-cell imaging dishes and chambers

Live-cell imaging can be conducted on standard thick-bottomed plastic tissue culture plates using long working distances lenses but at the cost of sensitivity and resolution. This can be well suited for transmitted light (e.g. phase contrast) cell tracking, cell counts or visualization of cell morphology, but less suitable for confocal fluorescence applications. Choose #1.5-thickness coverslip-bottom chambers or dishes that enable the use of higher NA (> 0.5) objective lenses. These range from 35mm dishes (e.g. MatTek, Wilco, World Precision Instruments), and 2, 4, or 8-well chambers (e.g. Labtek, Ibidi, Eppendorf), to devices for chemotaxis and flow experiments (e.g. Ibidi). Multi-well plates (24, 96, and 384-well) are also available with glass coverslip bottoms (e.g. Corning, Eppendorf, Ibidi). Particularly sensitive cell types, such as stem cells or neuronal cultures, may not adhere well to

glass substrates. In this case, coat dishes with e.g. polylysine or collagen (or purchase them pre-coated). Alternatively, optical-quality polymer dishes (Ibidi) can be used. Cells may also be grown on 25mm-diameter round coverslips and assembled into reusable chambers such as the Attotfluor® cell chamber (Thermofisher); however, these may be more prone to leaking compared to manufactured chambers and dishes.

#### *Quality control – live samples*

Live samples must be healthy before and during imaging: any cell stress will affect the behaviour of the cells and add to problems of phototoxicity during imaging. Even the motion associated with transporting cells (across campus for example) induces significant stress: consider incubating the cells for a few hours near the microscope before beginning an experiment. Inspect cells for normal morphology, proliferation and minimal cell death even before starting live-cell experiments and consider controls to assess the impact of the imaging you are undertaking.

## Choosing the right microscope

There is a wide diversity of 2D and 3D fluorescence microscopy techniques available, each with its own advantages and disadvantages. Often a correlative approach, applying several techniques to address the question from different perspectives, is required. Avoid the pitfall of '*the previous student in the lab used this microscope; therefore I should use this microscope*'. How, then, should you decide which microscope is best suited to your current experiment?

Start by consulting one of the increasing number of microscopy core facilities<sup>26</sup>, where you may find everything from slide scanners and high-end widefield microscopes to confocal (both CLSM and SD), multiphoton, and super-resolution microscopes. Core facility staff have extensive experience matching researchers to the most suitable microscope. The increasing prevalence of networks of microscopy experts and nationwide/regional infrastructures (e.g. BioImaging North America, Euro-BioImaging, BioImaging UK, SingaScope and Microscopy Australia) has further broadened the selection.

When selecting a microscope, the key question is "What is the biological question and what must you measure to answer it?" Consider the following factors:

- **Sample Viability:** Is the sample alive or fixed? In fixed-cell imaging fluorophores can photobleach (especially in high resolution z-stack image collection) and in live-cell imaging, fluorescence excitation leads to phototoxicity, in both cases reducing sample viability. Choose a technique and microscope configured to be light efficient and sensitive, which enables excitation intensity to be minimized. Researchers must provide evidence that cells remain viable during and after imaging, minimally through the maintenance of normal morphology and activity and continuation of the cell cycle<sup>27</sup>. Inverted microscopes are often best suited for live-cell imaging chambers to control environmental conditions including sterility, temperature, CO<sub>2</sub> and humidity.
- **Speed:** For live-cell imaging, the speed of image acquisition must match the dynamics of the process being studied. Even with fixed samples, acquisition speeds can become important when capturing many thousands of images (e.g. for an image-based screen), large 3D z-stacks or very high-resolution images. Higher sensitivity instruments can enable faster acquisition without increasing the excitation light intensity. A typical confocal acquisition time is hundreds of milliseconds to several seconds per fluorophore channel, but modern instruments can be equipped to capture hundreds of images per second for more rapid data collection and for visualizing rapid dynamic processes.
- **Resolution:** How small are the features that need to be resolved? The lateral (XY) resolution of a fluorescence microscope depends on the wavelength ( $\lambda$ ) of light and on the Numerical Aperture (NA) of the objective lens<sup>28</sup>. Rayleigh's criterion is a rule-of-thumb for estimating the smallest features that can be resolved laterally:

$$R_{xy} = \frac{1.22\lambda}{2NA} \quad \text{Equation 1}$$

For example, a high-NA oil immersion objective (NA = 1.4) can image a green fluorophore ( $\lambda \sim 500$  nm) with  $\sim 200$  nm lateral resolution. Axial (Z) resolution is about 2 to 3 times worse than lateral resolution ( $\sim 400$  to  $600$  nm in this example).

- **Contrast:** The ability to 'see' features of interest against the background is vital. Confocal microscopes and related optical sectioning techniques lower the background by excluding out-of-focus light, thereby providing better contrast.



- **Depth Penetration:** Due to absorption and scattering in most biological specimens, depth penetration is generally limited to 50-100  $\mu\text{m}$  for confocal microscopes, and perhaps twice that for multiphoton excitation (see *Alternative optical sectioning methods*), unless tissue clearing techniques are employed<sup>2</sup>. Shorter wavelength light (blue/green) experiences higher absorption and scattering than longer wavelengths<sup>29</sup>, so choose red or far-red fluorophores for deeper imaging but at the cost of resolution (Figure 3).

Every microscope comes with trade-offs between these parameters. For example, there is often a tendency to prioritize higher image resolution inappropriately. In a higher-resolution image each pixel captures a smaller area, resulting in less signal per pixel. This may require increasing the excitation light intensity or pixel dwell time, thereby increasing photobleaching and phototoxicity or decreasing the acquisition speed. Higher resolution images are also larger, requiring more storage space. The goal is to have the appropriate level of resolution to answer the question at hand. In Figure 5a, the parameters listed above are plotted on the axes of radar plots to visualize how different microscopy techniques perform relative to each other. These are generalized examples – the actual performance of each microscope depends on its exact configuration and settings (Figure 5b). Nevertheless, these microscopy techniques will be examined one-by-one to offer guidance.

#### Widefield Fluorescence Microscope (WF)

When imaging a cell monolayer, a well-equipped modern widefield fluorescence microscope may actually be the most suitable choice. Widefield microscopes are very light efficient and therefore preserve sample viability better than most confocals. Newer sCMOS digital cameras are incredibly fast, capable of capturing hundreds of images per second, given sufficient signal. The lack of optical sectioning results in poorer contrast and depth penetration, prohibiting the discrimination of signal arising from different structures in the axial (depth) direction (e.g. nucleus and plasma membrane). This renders widefield microscopy generally unsuitable for colocalization analysis<sup>30,31</sup>. Quantitative deconvolution algorithms<sup>32</sup> can improve contrast for samples less than  $\sim 20\ \mu\text{m}$  thick or sparsely labeled specimens, provided a z-stack is acquired.

#### Spinning-Disk Confocal Microscope (SD)

SDs are generally optimized for live-cell imaging, using fast, sensitive cameras, and they excel at this application. Depending on the camera, speed and sensitivity may come at the expense of resolution and/or field of view. Older instruments employed 512 x 512 pixel (i.e. 0.25 megapixel (MP)) EMCCD cameras, with very high sensitivity but low resolution and a small field of view, but newer SDs may use higher resolution large field of view 2.5 MP sCMOS cameras. Most SDs are limited to imaging 1 colour (single camera system) or at most 2 colours simultaneously (dual camera system or single camera plus image splitter), such that multi-channel acquisition is rate-limited by filter/mirror movements. Since the SD has many pinholes operating in parallel, images are collected much faster but the scattered light prevalent in thicker samples may be imaged through the wrong pinhole (called “pinhole crosstalk”), resulting in a loss of contrast when compared to CLSM<sup>33</sup>. Some newer SDs have multiple pinhole size and spacing options to counter this phenomenon.

#### Confocal Laser-Scanning Microscope (CLSM)

A CLSM provides superior resolution, depth penetration, and contrast compared to WF and SD but is generally less well suited to live-cell imaging (Figure 5a). CLSMs must scan a single laser beam across the specimen, generating the image one pixel at a time. Consequently, a CLSM is slower and requires higher laser power (lowering the score for sample viability). However, with modern hardware and the highly configurable software typical of CLSMs, the same instrument can be optimized for different experimental requirements. Figure 5b demonstrates how the key parameters for a CLSM,

on the same instrument, can be specifically optimized for either live-cell imaging (faster speed) or contrast (improved depth penetration and resolution). Faster resonant-scanning mirrors, which sweep the beam across the specimen 10 times faster than traditional galvanometer scanning mirrors, are closing the gap between CLSM and SD in terms of speed. CLSMs often have three or more detectors that can operate simultaneously, enabling rapid, multicolour live-cell imaging, particularly when combined with resonant-scanning. New array detectors, such as the Airyscan (Carl Zeiss), offer increased sensitivity and resolution simultaneously<sup>34</sup>. Higher sensitivity GaAsP or hybrid detectors more than double the efficiency of conventional PMTs, allowing the laser power to be reduced. Using a larger pinhole also improves sensitivity and live-cell viability, while sacrificing z-resolution (see *Setting Up the Microscope*). Indeed, the CLSM's flexibility to adapt to the experiment at hand makes it the workhorse of most microscopy core facilities<sup>33</sup>.

### Alternative optical sectioning methods

Other microscope techniques can offer a variety of advantages over confocals, depending on the experimental needs and biological questions. A multiphoton microscope uses a pulsed infrared laser to achieve greater depth penetration, and since the excitation volume of  $\sim 1$  femtoliter is confined to a discrete location in x,y and z it enables localized 3D photoactivation/photobleaching<sup>35</sup>. Total Internal Reflection Fluorescence (TIRF) microscopy restricts excitation to  $\sim 100$  nm adjacent to the coverslip surface, thus providing superior axial resolution and signal-to-background compared to confocal techniques, particularly for fluorophores in or near the plasma membrane<sup>36</sup>. Super-resolution microscopes offer improved resolution down to  $\sim 110$  nm (Structured Illumination Microscopy, SIM),  $\sim 50$  nm (STimulated Emission Depletion, STED), or even  $\sim 20$  nm (Single Molecule Localization Microscopy, SMLM), but the resolution enhancement typically comes at the expense of speed, sample viability, and depth penetration and often requires special fluorophores and/or buffers<sup>37,38</sup>. For thick specimens such as whole zebrafish embryos or cleared tissues and organs<sup>2</sup>, lightsheet microscopes offer advantages in both speed and low phototoxicity, though typically at the cost of resolution<sup>39,40</sup>.

### Quantitative measurements of dynamics and interactions

Confocal microscopes (CLSMs in particular) are excellent platforms for a host of quantitative techniques for measuring molecular dynamics and interactions<sup>41</sup>. Fluorescence Recovery After Photobleaching (FRAP)<sup>42</sup> measures diffusion or binding of fluorescently tagged molecules by purposely photobleaching a region with high laser power, and subsequently recording the recovery of fluorescence to that region as unbleached fluorophores exchange with the bleached ones. Photoactivation<sup>43</sup> or uncaging uses the opposite approach: dark molecules are converted to fluorescent ones with laser illumination, whose motion can then be observed. Fluorescence Correlation Spectroscopy (FCS)<sup>44,45</sup> and Raster Image Correlation Spectroscopy (RICS)<sup>46</sup> measure concentrations, diffusion of fluorophores, and other fluorophore behaviour such as blinking (FCS only). These complementary techniques, which require rigorous acquisition and analysis<sup>47</sup>, each have their own advantages for measuring cellular dynamics.

Measuring interactions between molecules is not a trivial task. Colocalization analysis does not confirm interactions between molecules, only that the two fluorescent tags are both located within a few hundred nanometers of each other (i.e., within the resolution limit of the microscope). Fluorescence Resonance Energy Transfer (FRET)<sup>48</sup> experiments can be used to demonstrate that fluorescent probes on targets of interest are within 5 to 10 nm of one another, which typically confirms protein interaction. The major disadvantage of FRET experiments is that a negative result could arise simply from the fluorescent tags themselves being further than 10 nm apart, even if the two protein domains themselves are interacting, or due to inappropriate orientation of the dipoles<sup>49</sup>.

Alternatively, imaging co-dynamics of protein complexes is highly indicative of protein interactions. As an extension to FCS, dual-colour dynamic imaging of two molecules of interest with Fluorescence Cross Correlation Spectroscopy (FCCS)<sup>50,51</sup> can be used to show co-dynamics. As a control, mutated proteins that do not show co-dynamics can provide strong evidence for direct protein binding. Finally, the Proximity Ligation Assay (PLA) has been developed to identify proteins/targets within ~40 nm of one another (suggestive of interaction) in fixed samples using antibody staining protocols<sup>52</sup>.

## Planning your experiment

### Pilot projects

Experimental design, data acquisition, and data analysis may seem to be consecutive steps in the experimental pipeline, but in fact they form a continuous feedback loop, each one informing the others. Lessons learned in the “final” step of data analysis can inform the overall design of an experiment, as well as the parameters used to acquire the data. A pilot project is an excellent way of starting this feedback loop in motion. It provides an opportunity to validate a quantitative imaging pipeline on a smaller scale before investing time, energy, and resources in a full-scale experiment.

A good pilot project involves performing every step of the process all the way through to generation of a final output graph. Running through the entire process identifies issues that require fine-tuning. Live-cell imaging involves compromises between spatial resolution, temporal resolution, signal intensity, and sample viability (the so-called “tetrahedron of frustration”). Unanticipated limitations (e.g. poor cell viability or lower intensity than expected) may necessitate adjustment in cell preparation or experimental parameters. Small changes in image acquisition parameters often result in data that is far more readily analyzed and potentially meaningful. For example, a small reduction in spatial resolution could result in a critical improvement in signal intensity, allowing easier segmentation and quantification. A pilot project also presents opportunities to observe unexpected phenomena, thereby increasing the ways in which an experiment can be analysed. For example, a change in localisation of fluorescent vesicles might be accompanied by an unexpected change in fluorescence intensity, or vice versa. Preliminary measurements are often required in drug-treatment experiments in order to determine the time point at which maximum effect is obtained. A pilot project also provides an initial estimate of the strength of the predicted biological effect, which helps you to estimate the number of samples that must be imaged in order to measure an effect. For example, if, in the pilot study, there is only a subtle change of 25% between two conditions, then 100 or more images may be needed for each condition to measure the change with statistical confidence; but if there is a two-fold change, 30 images may suffice (see *Statistics* section).

### Removing bias

Bias can taint any experiment including those that use confocal microscopy<sup>53</sup>. If Slide #1 is expected to have brighter labeling than Slide #2, your eyes might be drawn (either consciously or subconsciously) to capture brighter cells in Slide #1. When reviewing images, you might think of reasons (“excuses”?) to throw away images that skew the average intensity in an uncomfortable direction. If microscopy experiments are to yield truths, we must find ways of preventing our human biases from affecting how we capture and analyze confocal images.

In clinical research, the gold standard is to conduct a randomized, double-blind, placebo-controlled study<sup>54</sup>. Both researcher and subject are blind to whether the subject is receiving the treatment or the placebo: blinding ensures that subjective measurements, such as improvement in patient-reported pain, are not influenced by expected or desired outcomes. Most confocal microscopy users, however, will prepare, image and analyze their own slides, which often includes subjective

choices such as which fields of view to capture or what threshold to set for counting positively-labeled cells. Consider asking a colleague to label the slides so that you are blinded to control and experimental conditions until acquisition and analysis is complete.

When imaging tissue sections, remove the field-of-view selection bias by imaging and analyzing entire structures or even the entire tissue. Confocal microscopes equipped with motorized stages can be set up for tiling-and-stitching large regions in 2D or 3D. Since 3D Z-stacks can be time consuming, consider limiting the acquisition to 20x or even lower, if that resolution is sufficient. To quantify labeling in very large samples such as whole organs, tissue clearing<sup>2</sup> combined with light-sheet microscopy<sup>39,40</sup> may be a better choice. However, be prepared for extremely large datasets: a single whole-organ light-sheet dataset can be as big as 1TB, requiring special computer infrastructure for viewing, rendering, and quantifying the signal. Alternatively, physically slicing thin tissue sections (5-15  $\mu\text{m}$ ), enables them to be scanned on increasingly common whole-slide fluorescence scanners. Regardless of whether it was scanned on a widefield slide scanner or tiled on a confocal microscope, whole-slide analysis software (e.g., Halo<sup>®</sup> by Indica Labs, VisioPharm<sup>®</sup>, or the new freeware QuPath<sup>55</sup>) can quite easily quantify nuclear, cytoplasmic, and membrane labeling across an entire tissue (among other features). Traditionally, whole-slide analysis was complicated by the fact that recognizing structures in the tissue still had to be done by a qualified pathologist, but new machine learning-based pattern recognition classifiers make it easier to separate tissue types (e.g. tumour versus stroma) by training the algorithm with example regions.

In the field of Neuroscience, stereology rose to prominence from 1970 to 2000 as an unbiased and accurate way of estimating geometrical features (e.g. number, length, volume, etc.) in 3D structures from 2D brain slices<sup>56</sup>. Stereology, which can be performed on both widefield and confocal microscopes, adds a software component that provides random, systematic sampling according to rigorous statistical principles. However, stereology will only be successful if unbiased sampling is achieved, which starts with the very first decision on how to section the tissue. As whole-slide scanning and 3D imaging and analysis techniques have developed, the use of stereology has declined<sup>57</sup>. While stereology may not be necessary for most experiments, confocal microscope users often do not put enough thought into rigorous sampling of their 2D or 3D specimens.

## Setting up the microscope

With so many components to configure, setting up a confocal microscope for quantitative image acquisition can be a daunting task! This step-by-step guide takes you through the most important aspects of configuring the microscope.

### Stage inserts

It can be surprisingly difficult to mount your samples flat and stable on the microscope. A variety of interchangeable stage inserts are usually available: check that one is properly installed and leveled. Mounting the sample itself into the stage insert, to be held firmly and presented exactly perpendicular to the optical axis, can also be challenging. If the sample is even slightly tilted, the image will appear brighter on one side than on the other and this brighter region will shift across the image as you move through focus. For imaging slides on an inverted microscope, ensure that only the edge of the glass slide is resting on the insert (not the coverslip or label). If you are using coverglass chambers together with high-NA objective lenses, keep the center of the objective lens away from the edge of the chamber to avoid the lens hitting the stage insert: unfortunately, you won't be able to image right to the edge of these chambers.

### Selecting an objective lens

One of the most critical decisions is choosing the ideal objective lens for the specific experiment (Figure 6 and Table 1). The most significant factor in determining both sensitivity and resolution is not the magnification (see Supplementary Figure 1), but the Numerical Aperture (NA) of the objective, which is prominently indicated next to the magnification on the lens (e.g. 20x/0.8 NA). The NA is defined by the half-angle of the cone of light that is focused or collected by the objective ( $\theta$ ), and the index of refraction of the mounting medium ( $n$ ):

$$NA = n \cdot \sin\theta \quad \text{Equation 2}$$

Looking back at the resolution equation (Equation 1) we see that doubling the NA, even at the same magnification, results in twice the resolution (i.e., features that are twice as small can be resolved). Perhaps even more importantly, when the NA is doubled the amount of light collected from the sample is 4 times higher: high-NA objective lenses are crucial for capturing fluorescence efficiently. However, be aware that the laser is focused to a smaller spot so the irradiance (laser power density) during excitation is also higher, which has consequences for photo-bleaching and sample viability. Since the NA of an objective depends on the refractive index of the immersion medium, an oil immersion lens ( $n_{\text{oil}} = 1.518$ ) can have a higher NA than a water lens ( $n_{\text{water}} = 1.33$ ), while a dry lens ( $n_{\text{air}} = 1.0$ ) will have even lower NA. Should oil immersion lenses always be used for fluorescence microscopy? No. First, the high NA of oil immersion lenses comes with the trade-off of a shorter working distance (WD-distance from the objective front lens to the closest coverslip surface when the sample is in focus), typically <200  $\mu\text{m}$ . The short WD makes it only compatible with imaging through a coverslip (Figure 6c), and limits the depth of imaging into the sample. Tissue culture microscopes will instead be equipped with lower NA, longer WD dry objectives (Figure 6b) that are adequate for seeing cells through thick tissue culture plates or flasks, but with lower resolution and sensitivity. Special long WD water or glycerol immersion objectives maintain reasonably high NA but at the expense of high cost. Second, mismatch between the refractive index (RI) of the immersion medium and that of the sample can cause severe spherical aberration artifacts, and the severity increase deeper into the sample. Thus, when imaging a thick biological specimen, with a RI that is very different from standard immersion oils, or a cell monolayer in aqueous medium, the advantages of using a water, glycerol or silicone oil immersion lens to attain a better RI match may outweigh the disadvantage of slightly lower NA. Compare different objectives to see which works best. If the

objective has a correction collar, it should be set at the position for the corresponding immersion medium, coverslip thickness and/or temperature, or better still, adjusted while imaging (assessing by image sharpness and brightness) to compensate for the thickness variation of even a #1.5 coverslip (160-190  $\mu\text{m}$ ). Third, consider what resolution is needed for the experiment: a 20x/0.8NA dry objective (Figure 6a) gives a field of view as high as 0.5 x 0.5 mm (nearly 10 times that of a 63x objective lens), while still providing a resolution of 400 nm, which is more than adequate for quantifying nuclear intensities, for example. This high-NA dry objective may also be an excellent choice for long-term, medium resolution, multi-location timelapse imaging of live cells. Unlike most camera-based microscopes (including the spinning disk), the optics within a CLSM allow for true optical zoom during acquisition (rather than digital zoom on the acquired image), so that there is no advantage, for example, in using a 100x/1.4 objective lens over a 63x/1.4 objective coupled with adequate zoom.

Once your objective lens is selected, ensure it is clean (dried oil left on the lens by a previous user is a common problem). If required, clean it with specialized lens tissue (avoid waxy “lens paper”) or cotton-tipped applicators and an appropriate lens cleaning fluid. The front lens of an objective lens is often concave so care and repetition are required to clean them properly.

### Finding the cells

Most confocal users (present authors included) use the binoculars to search the slide for representative cells or the most meaningful features in the tissue. Using transmitted light (such as Differential Interference Contrast (DIC)) to find the areas of interest will reduce photodamage to the sample, but many users find it easier to use widefield fluorescence visualization for its higher contrast or its ability to rapidly locate specific fluorescently labeled features, cells with reasonable protein expression levels, or dual-expressing cells. However, the sample is prone to rapid photobleaching during this preliminary observation! Figure 7 shows a field of view that was observed with a typical metal halide lamp (X-Cite 120Q, Excelitas) for just 60 seconds on the maximum intensity setting while centering and focusing on the cells. Even the relatively stable fluorophore Alexa Fluor 488, mounted with Prolong Gold antifade mounting medium, bleached by 50% in just 3s, and by 75% in 10s. How much time spent on each field of view before even beginning to collect confocal images can destroy a substantial (and unknown) amount of fluorophore.

To minimize photobleaching:

- Adjust the lamp to its lowest setting, usually around 10% (use neutral density filters to go even lower for live cells), and allow your eyes to adjust to the dimmer setting (turn down the room lights). This single act will lower the photobleaching curve dramatically.
- Close the fluorescence shutter the second you finish observation.
- For very sensitive experiments, use transmitted-light contrasts to focus and find the cells.
- Establish the acquisition settings on a test region and then acquire experimental data from an unexposed area.

### Setting up for live-cell imaging

For confocal imaging of live cells<sup>19</sup>, maintaining an appropriate physiological environment (temperature, pH, and humidity) is essential. Either stage top incubators or large microscope enclosures can be used for this. When using immersion lenses with stage-top incubators, a separate objective heater must be employed to prevent the objective from acting as a heat sink. Humidification of environmental control chambers is often inadequate: to avoid changes in

osmolarity due to evaporation, place wet tissues in the chamber or fill empty wells of a multi-well plate with water. Since focus drift is endemic on microscope stands, use hardware autofocus devices (such as Zeiss's Definite Focus, Nikon's Perfect Focus, Leica's AFC, or Olympus's ZDC) to ensure the focus remains stable during timelapse experiments.

Most cells or tissues are never exposed to light in their natural physiological environment, making them particularly sensitive to fluorescence illumination. One side effect of photobleaching is the production of highly reactive and damaging oxygen radicals that cause phototoxicity. Consider whether the live imaging question requires confocal microscopy at all: many experiments such as cell tracking, proliferation or wound healing<sup>58</sup> can be addressed using transmitted-light techniques (e.g. Phase Contrast or DIC) on a widefield microscope. Shorter wavelength light is considerably more phototoxic to cells than longer wavelengths. Avoid blue-emitting dyes for live-cell work, and, if possible, use the red channel for locating cells in multiply-labelled samples.

### Configuring and optimizing fluorescence channels

The excitation and emission spectra for a typical combination of 4 fluorophores is shown in Figure 8a. On a CLSM, setting up fluorescence channels for multiple fluorophores from scratch can be bewildering. Since many of the spectra overlap, what is the best way to collect each fluorophore's emission as efficiently as possible, while avoiding crosstalk (also called "bleedthrough") from the other fluorophores? Luckily, most CLSMs have a wizard to help get you started: simply select the fluorophores from a list, and choose one of the various multi-channel strategies (Figure 8b-d). Many CLSMs have 3 or more detectors and allow illumination with multiple lasers simultaneously. However, to avoid crosstalk, simultaneous scanning should generally be avoided (see Figure 8b). The nuclear stain DAPI has a particularly broad emission that extends all the way to 600 nm (red), and will show up in the other detector channels in simultaneous scan mode (Figure 8e, f, g). Simultaneous scanning should only be used when speed is of the essence in the fastest of live-cell imaging experiments (post-acquisition crosstalk corrections should then be applied). A second strategy looks tempting (Figure 8c), here imaging two colours with low overlap (DAPI and AF568) simultaneously and the fluorophore between them (AF488) on a separate pass of the lasers. Unfortunately, this schematic offered by the wizard can be misleading because it assumes all of the fluorophores have equal intensities: if DAPI is much brighter than AF568, there will still be substantial bleedthrough of DAPI emission into the AF568 channel. The safest (though slowest) strategy is to image each channel separately (Figure 8d), with one laser and the corresponding detector turned on at a time. It is preferable to switch channels line-by-line rather than frame-by-frame to avoid temporal delays between channels. The wizard configurations are only a starting point and are rarely optimal. For example, some CLSMs have fixed emission filters, but the confocal pictured here has adjustable filter "gates" that could be further narrowed or broadened to improve specificity or sensitivity respectively. To ensure reproducibility between experimental replicates, configuration files can be saved and loaded during subsequent sessions, or settings can be applied from a saved image. For an SD equipped with just one camera and fixed filters, the configuration is straightforward – simply choose the 3 fluorescence channels and there is likely a single corresponding configuration.

Sometimes crosstalk is unavoidable, for example, when using CFP and YFP for a FRET study, or when using more than 4 fluorophores at once. Spectral unmixing<sup>59</sup> allows the separation of several fluorophores with overlapping spectra post-acquisition. Caution is required with this approach: good results will require single-labelled controls for each fluorophore to measure accurate reference spectra, and it is critical to balance the staining intensity for each fluorophore and ensure the separation is artifact free.



Image the brightest sample first, such as a positive control. Optimize the intensity of each fluorophore, turning on just one channel at a time to prevent photobleaching of the others. Start with a low laser power, around 1% to 5% of the maximum, to minimize photobleaching. On a CLSM, start with setting the pinhole diameter to 1 Airy Unit (AU), which is typically a good balance between optical section thickness and signal-to-noise ratio (SNR)<sup>60</sup>. The pinholes on a spinning-disk are usually a fixed size but several options may be available. Start a live preview, and adjust the detector sensitivity (sometimes called Gain or High Voltage (HV), on CLSMs) or camera exposure time (SD) until an image is observed. Adjust the focus to select the brightest focal plane. If the image is too noisy (CLSM) or the exposure time is too long (SD), increase the laser power and readjust the detector or camera accordingly while monitoring photobleaching. Cameras on SDs may also have sensitivity settings that can be adjusted, but again with an increase in noise. To further reduce noise in the image on a CLSM, slow down the scanning speed so that the pixel dwell time is at least 1 to 3  $\mu\text{s}/\text{pixel}$ , and/or use line or frame averaging (typically 2 to 4 averages). To optimize the intensity, confocals have a special lookup table (colour map) to reveal areas on the image with pixels that are too bright ("saturated") and pixels that are zero intensity (Figure 8h-k). If any type of quantification is to be performed, it is imperative to avoid any "clipping" of either the brightest or weakest pixels. Acquire the raw data across the entire intensity range and use digital contrast adjustments or background intensity subtraction to reveal the features of interest, applying the same adjustments to all images to be compared. To maintain the same settings, the settings chosen must encompass the full range of intensities across all specimens. Using a higher bit depth setting (16-bit, rather than 8-bit) gives a greater number of gray levels between black and white, which may allow the capture of subtle intensity differences in both the brighter and weaker features of a specimen. If it is impossible to image a bright and a weak sample with the same settings, adjust the laser power and acquire a second set of corresponding images. Take note of the two laser power settings, or more rigorously measure them with a power meter through the objective lens, so the difference can be taken into account during analysis.

### Optimizing spatiotemporal parameters

Having adjusted the intensity for each fluorescence channel, next establish the set of parameters that affect the size and shape of the image and the speed of acquisition. The default zoom for a CLSM (zoom 1) will set the confocal to scan a square area roughly two-thirds of the way to the edge of the binocular field of view. The default image size of 512 x 512 pixels (0.25 megapixels) is generally insufficient to preserve the details of the image, resulting in a pixelated appearance and making it difficult to analyze or generate high-quality figures for publication. Instead, use at least 1024 x 1024 pixels (1 MP), and/or zoom in to a smaller region of interest. For maximum resolution, the pixel size should be 2 to 3 times smaller than the optical resolution of the objective lens as calculated by Equation 1 – a condition called "Nyquist sampling". Some CLSM software has an "optimal" button that, given the current objective lens and zoom, calculates the number of pixels you need to achieve Nyquist sampling. Since a CLSM scans one pixel at a time, if the maximum field of view is selected (zoom 0.7) with Nyquist sampling (say, 4096 x 4096 pixels), it may take several minutes to acquire a single (2D) confocal image. Some compromise may be required to balance the quality of the image with the acquisition time: if the smallest structure that must be resolved is larger than the objective resolution, the Nyquist calculation may be applied to the structure size instead. The scan speed is also adjustable: aim for a pixel dwell time (reported in the software) of about 1  $\mu\text{s}/\text{pixel}$  to prevent noisy images. While SDs lack the flexibility of CLSMs to scan any size field of view, if the SD has multiple cameras with different resolutions, switching between cameras will have the effect of zooming and/or changing the pixel resolution.



Having optimized a single XY (lateral) image, apply the same principle of sampling to the Z (focus) dimension. The optical sectioning thickness depends on the NA of the objective and the size of the pinhole(s). On a CLSM, the software should report the slice thickness as the pinhole size is changed, and then automatically calculate the optimal z-interval based on the Nyquist sampling theory of 2 to 3 overlapping z-steps per optical slice thickness. SDs may not indicate the required z-step for Nyquist sampling, requiring prior knowledge of the maximum achievable z-resolution with a given objective lens. As a rule of thumb, the slice thickness is about 3 times the Nyquist pixel size in x,y and will be  $\sim 1\text{ }\mu\text{m}$  for a 63x/1.4NA oil immersion objective on a SD.

The CLSM is particularly adaptable – recall Figure 5b, where we showed that the same CLSM can be optimised for either fixed or for live-cell applications. When contrast is the priority, keep the pinhole at the theoretical optimum 1 AU and consider increasing the laser power, slowing down the scan speed, and averaging 2 to 4 images while reducing the detector gain. The radar plot (Figure 5b, red curve) shows that this combination benefits depth penetration and resolution, but particularly compromises the speed of acquisition (which can climb to several minutes for a small z-stack) as well as sample viability. When optimizing for live-cell imaging (Figure 5b, green curve), suggested settings for optimal speed and lower phototoxicity include simultaneous instead of sequential channel acquisition, the use of higher sensitivity GaAsP or hybrid detectors, lower laser powers, faster scan speeds (with regular galvanometer or resonant scanners), a pinhole diameter greater than 1 AU, and increased gain. These parameters sacrifice contrast, depth penetration, and resolution but make live-cell imaging possible on the CLSM.

## A quagmire of unexpected instrumentation issues

With a new understanding of how to prepare optimal samples and set up the confocal microscope with appropriate settings to avoid photobleaching, saturation or cross-talk between channels, you feel ready to acquire quantitative data. You plan to image all of your slides in one 3-hour session, using the same settings. ***What could possibly go wrong!?***

### Problem #1: Non-uniform illumination

Confocal manufacturers try to configure and align the microscope with uniform illumination intensity across the field of view, but the lack of consistent design specifications leads to variability. Figure 9a-c shows a particularly bad example from a 20x/1.0 NA intravital imaging objective, which has an unusually wide field of view given its high NA and long working distance (1.9 mm). At a zoom of 1, the intensity varies by 40% between the center of the image and the corners, and a massive 60% using the minimum scan zoom of 0.7x. This uneven illumination would lead to a 60% difference in the intensity of the same cell depending on where it was positioned in the field, adding considerable (and systematic) error to any intensity measurements. Most CLSMs have at least a 10% variation in their illumination (corner to center). Users can image a uniform fluorescence sample and apply a software correction (called “flat-field” or shading correction). However, most users don’t have these special slides, and since the illumination profile changes over time they need to be re-measured regularly which is often not practical. Instead, manufacturers should be encouraged to adopt a standard of < 5% illumination non-uniformity at zoom 1.

### Problem #2: Focus drift

Many confocal microscope stands experience significant focus drift over time. This is revealed by an increasingly blurry image on a widefield fluorescence microscope, but on a confocal microscope, the rejection of out-of-focus fluorescence leads to a change in intensity as well as different features appearing in the image. Figure 9d shows how the focus on a new CLSM takes several hours to stabilize. This might be anticipated when turning on an environmental chamber, since small changes in temperature expand the metal microscope stand, but not for this dataset recorded at a stable room temperature using a fixed slide. Tests of 8 microscope stands by 4 different manufacturers revealed varying degrees of focus drift for all, with some never stabilizing, but continually drifting down with gravity. We encourage the manufacturers to adopt a standard whereby the microscope must stabilize to less than 1  $\mu\text{m}$  focus drift/hour within the first hour after initialization, and to test and replace faulty focus drives on a regular basis.

### Problem #3: Laser instability and power variation

Laser instability is perhaps the most insidious and common (though largely unappreciated) instrumentation issue for CLSMs. Figure 9e shows how one model of femtosecond laser (used for multiphoton microscopy) performs during the first hour of operation after being keyed on from standby mode. The laser starts emitting after about 5 minutes, but there is a dramatic change in intensity when the cavity automatically re-optimizes 30 min after start-up. Even worse, over the next hour if the laser is simply tuned to a new wavelength, it again automatically re-optimizes causing the power to change by a further 15%, but if the wavelength is not changed, it maintains the lower power for the duration of the imaging session. This model of laser was clearly designed to favour maximum power rather than reproducibility. As another example of laser instability, Figure 9f shows continual variations in laser intensity of more than 12% for one visible laser on a 4-laser CSLM system, adding a 12% uncertainty on all intensity measurements. This type of fluctuation, which requires a rarely-performed time-series power measurement to detect, is common as diode lasers age, but can also occur on new lasers. Finally, Figure 9g shows how the power of the same laser line (488 nm) can vary tremendously between similar instruments. All of these fluctuations and intensity

changes, undetected by the user, will prevent meaningful, quantitative comparisons from one system to the next, from one day to the next, or even from the start to the end of a single imaging session. Thus, regular quality control sessions and good test standards are currently required for rigorous quantification. Since most users do not have access to a power meter, or the time to remove the sample and check frequently for fluctuations (particularly impossible during a time-lapse session!), there is a need for more convenient ways to measure the exact power of each laser. Manufacturers should be encouraged to incorporate optical sensors into confocal microscope scanheads, and provide real-time closed-loop feedback to maintain steady laser powers during an imaging session. Finally, we implore the manufacturers to abandon the uncalibrated illumination unit of percent-laser-power, and instead provide a real-world unit of power (mW), or even irradiance (illumination density) in  $\text{W/m}^2$ . This will enable the confocal community to establish guidelines for reasonable irradiance values under various experimental conditions (fixed cells, live cells, etc.).

## Analyzing and presenting quantitative images

Having spent considerable effort acquiring quantitative confocal images, it's essential to process and analyze the images in a manner that does not jeopardize their quantitative nature. Although the confocal vendor's acquisition software may include image processing and analysis features (with various degrees of sophistication), most users turn to comprehensive third-party software. The freeware Fiji ImageJ is an excellent choice<sup>61,62</sup>. Fiji is available on all operating systems (MAC, PC, Unix), and thanks to the integrated BioFormats Importer it reads most proprietary image formats (including important metadata such as the pixel size) without any conversion<sup>63</sup>. Multiple channels can be displayed individually, as a composite, or split out into separate image windows. Timeseries and z-stacks are treated as a series (called "stacks") in a single window, with a slider to move between the individual images. From filtering (e.g., Gaussian smoothing) to thresholding (including 17 auto-thresholding algorithms), to measuring areas and intensities of objects ("Analyze Particles"), Fiji has the basics covered and much more. Furthermore, Fiji's open source architecture has led to the creation of hundreds of plugins by the community of users, including sophisticated ones such as Trainable Weka Segmentation<sup>64</sup> (using texture and machine learning to select and classify tissues or cells) and Trackmate<sup>65</sup> (automated, semi-automated, and manual tracking of single particles). Repetitive processing and analysis steps can be automated using macros with minimal programming proficiency required: simply record macro commands while analyzing one image, then edit and run the macro on subsequent images. To get started with Fiji, refer to Arena *et al*<sup>62</sup>, search the internet for online tutorials, and/or consult staff at your local microscopy core facility.

CellProfiler<sup>66</sup> is another freeware analysis program, specifically tailored to quantifying cell phenotypes. CellProfiler helps users create and execute analysis "pipelines", or sets of commands that are applied to an entire group of images. This can be particularly helpful for large volumes of images, such as those generated in a high-content screening experiment. Working with analysis pipelines can help ensure reproducible results: all images are necessarily processed identically, and the pipeline itself can be saved and scrutinized by supervisors or peers. A cloud-based version called Distributed-CellProfiler allows users to run analysis on thousands of images in parallel on Amazon Web Services. Commercial versions of cloud-based analysis pipelines are also beginning to emerge including Apeer (by Carl Zeiss), which has a free version for academic individuals. Cloud-based analysis platforms may be the best way to access computationally intensive new deep-learning algorithms for image restoration, such as de-noising and deconvolution, of fluorescence microscopy datasets<sup>67,68</sup>.

Some analysis tasks are not very convenient (or even possible) in Fiji ImageJ or CellProfiler. For 3D rendering and analysis, there are some Fiji plugins that may suffice including ClearVolume<sup>69</sup> and GIANI (developed by David Barry, CALM, Francis Crick Institute). However, depending on the application, more powerful commercial solutions may be more appropriate such as Imaris® (Oxford Instruments), Amira® (Thermofisher Scientific), Vision 4D® (Arivis), Aivia® (DrVision), or Volocity® (Quorum Technologies). The commercial platforms harness the full power of modern computers and their graphics cards, including GPU processing and acceleration. For imaging and analyzing large tissue sections, dedicated digital pathology software may be more appropriate than Fiji, such as Halo® by Indica Labs, VisioPharm®, or the new freeware QuPath<sup>55</sup>. These programs can handle huge tiled-and-stitched (or scanned) images efficiently. They have integrated machine learning classifiers to segment the tissues into types (such as tumour versus stroma) by the textures and patterns in the tissues rather than just intensity thresholds; and after classification there are algorithms for quantifying nuclear, cytoplasmic, and membrane labeling of cells among other metrics.

Regardless of what software you use and the kind of analysis you wish to perform, here are some key ways to maintain the integrity of a quantitative comparison<sup>70,71</sup>:

1. Always save the original, unmanipulated image in the microscope vendor's file format. If images are converted to TIFF format, the accompanying metadata (which records the imaging conditions - objective lens, laser power, pixel size etc.) may be lost unless you specifically use the BioFormats OME-TIFF format, developed by the Open Microscopy Environment (OME) consortium (<http://openmicroscopy.org>). JPEG images are suitable for presentations, but their lossy compression make them unsuitable for image analysis.
2. For presentations and publications, apply the same contrast settings to all images to be compared (typically raising the black level and lowering the white level). Non-linear adjustments, such as gamma changes that bring out medium intensity features, must be used with caution and always declared in a figure legend. Save a separate copy of the adjusted images and perform analysis on the original images.
3. All images that are to be compared must be processed in the exact same way. Furthermore, do not selectively edit one part of an image (e.g. brushing out a cell you don't like, or lowering the intensity for just one region of an image).
4. Touching up your images with photo editing tools (such as Adobe Photoshop®) is generally forbidden: the blemishes are also part of the data.
5. Confocal images are often noisy. Linear smoothing filters (such as the Gaussian low-pass filter) can be helpful (and are usually appropriate) before analysis or presentations.
6. Deconvolution can enhance 3D datasets (they have little effect on 2D images) by removing noise, boosting contrast, and improving resolution<sup>32,72</sup>. Use only rigorous image restoration algorithms, of which there are many kinds. Third-party programs such as Huygens Deconvolution (by Scientific Volume Imaging) have algorithms specifically optimized for different kinds of microscopes (CLSM, SD, Multi-photon, STED, etc.), which may perform better than generic algorithms. Adjusting the deconvolution parameters (particularly the PSF, Signal to Noise Ratio and Background estimates), which have a profound effect on the outcome, takes some experience. For quantitative results, the same parameters should be applied across all images. Avoid using deconvolution on undersampled images, and watch for artifacts.
7. When setting a threshold for selecting positive cells for analysis, apply the same threshold to all images if possible. Alternatively, use the same autothreshold algorithm for each image – this may be necessary when there are variations in background levels.

## Statistics

When performing quantitative confocal imaging, it is important to determine at what point there is sufficient data to determine if there are statistically significant differences. Several good reviews on this topic are available<sup>73,74</sup>, and Nature has a collection of short articles on Statistics for Biologists (<https://www.nature.com/collections/qghhqm>). General guidelines are presented here using a specific example of imaging adhesion dynamics within cells. Adhesions are dynamic structures that act as traction points for cells and they form, grow and disassemble as the cell migrates. They can have many different sizes, shapes and dynamics (Figure 10a). In this example, the rate of adhesion assembly was calculated by measuring adhesion intensities over 10-30 minutes for multiple adhesions from multiple cells<sup>75</sup>.

It is important to repeat any experiment multiple times and ensure that the results are the same. Ideally, experiments should be repeated on different days and with all steps being independent between the replicates. Here, each experiment consisted of adhesion assembly measurements on 10 individual adhesions from 3 cells. The experiment was repeated 3 times so that there were measurements of 90 adhesions in 9 cells. Even with 30 adhesions measured for each experiment, variation within the experiment can be high (Figure 10b), 40% in this case, so three experimental replicates would be a minimum. Technical replicates are also important and consist of preparing the exact same sample multiple times in a single experimental run. This could mean making three, identically stained coverslips with cells plated, fixed and stained at the same time. Technical replicates are important for checking on consistency in sample preparation and can detect errors such as pipette errors, buffer pH or an old stock of reagent. Technical replicates should never be used to replace experimental replicates. Technical replicates were not performed in the adhesion example presented here.

Biological systems inherently show significant variability, hence the current boom in single cell analysis. Measuring many cells at once determines average behaviour while single cell data reveals subpopulations, outliers and interesting changes in underlying population distributions. Here, the cell-to-cell variation was high at 45%, while the variation between adhesion assembly rates within individual cells was only 35% (Figure 10c).

The high variability in biological samples makes it important to measure many cells/tissue sections/animals for proper characterization. A minimum of 30 independent samples is generally recommended, but may be difficult to achieve for experimental systems such as mouse models. In the adhesion example, a total of 90 adhesion assembly rates was measured from 9 cells (i.e. 10 adhesions per cell). Having measured just 3 adhesions would have been insufficient to characterize the population distribution (Figure 10d), but as few as 10 adhesions still allowed a reasonable estimate of the mean and the median of the distribution, even though adhesions with faster assembly rates were not well represented. The distributions for 30 or 90 adhesions were not very different unless a detailed analysis of subpopulations was of interest then the data set with 90 adhesions would be more valuable (Figure 10d).

It is becoming more widely accepted that individual data points should be displayed as box plots (Figure 10d), rather than simply as bar graphs (Figure 10e)<sup>76</sup>. Figure 10d and Figure 10e clearly show how the box plot provides the reader the ability to judge the quality and quantity of the experimental data directly, as well as plainly revealing outliers and clusters of data points within the distribution. Outliers should never be omitted from a box plot unless there is a flaw in the data collection or analysis, in which case formal tests to reject outliers should be applied<sup>73</sup>.

The number of data points collected for a given experiment becomes particularly important when trying to assess whether two data sets are significantly different (Figure 10f). The adhesion example shows assembly rates for control versus treated cells (growth factor treatment). Researchers typically use a t-test for this analysis and calculate the probability,  $P$ , that the means of the two data sets (e.g. control and treated) are not equal<sup>77</sup>. Data is then entered into a spreadsheet or scientific graphing package that automatically calculates the  $t$  and  $P$  values. A  $P$  value of  $< 0.05$  is often set as a cut off, meaning that if the experiment was repeated 100 times, the means of the data sets would be equal in 5 of those experiments (i.e., there is a 5% probability that the means of the data sets are not different). In general, a  $P < 0.05$  is accepted for biological systems with high variability but it is recommended to have a  $P < 0.01$  to be confident there is a difference. Confidence can be displayed with an indication of the number of asterisks for a comparison of two data sets, to aid the reader in determining whether they agree with the experimental conclusions (Figure 10f). Convention uses \* for  $P < 0.05$ , \*\* for  $P < 0.01$  and \*\*\* for  $P < 0.001$ .

The number of data points,  $n$ , plays a big role in the  $P$  value, leading to debate concerning the optimal value for  $n$ . In general, the choice of the  $n$  value and the  $P$  threshold for statistical significance depends on how confident the researcher wants to be in their conclusions. In the example given here,  $n$  could be 3 for the number of experiments, 9 for the number of cells, or 90 for the number of adhesions. The  $P$  value using  $n = 90$  adhesions was  $< 0.001$ ,  $P < 0.001$  for  $n = 9$  cells and  $P < 0.05$  for  $n = 3$  experiments. So even being cautious and using  $n = 3$  experiments there is a significant difference between the control and treated conditions.

When the means of the control and treated samples were compared for measurements of  $n = 3, 10, 30$  or 90 adhesions for control and treated cells (using  $n = \#$  of adhesions), the differences were not significant for only 3 adhesions each (control and treated), were significant with 99% confidence for 10 adhesions, and with 99.9% confidence for 30 or 90 adhesions. Therefore, 30 adhesions for each condition was considered the optimal number for this experiment.

## Conclusions

Is “quantitative confocal microscopy” an oxymoron? The more experience you have with confocal imaging, the more you realize just how many things can go wrong. Indeed, several experts in the field have opined that it is nearly impossible to obtain rigorous measurements of intensities in a confocal experiment. Yet, no reviewer will accept qualitative comparisons between microscopy images: you will most likely be asked to quantify them! This tutorial should provide you with the tools to do this.

The primary responsibility for rigor and reproducibility in confocal microscopy rests with the experimenter. However, core facility staff and microscope manufacturers can also play a role. Core facility staff should be allowed the time and resources to perform regular quality control sessions on the instruments, at a minimum measuring laser powers, cleaning objectives, and using a familiar, prepared slide to identify problems<sup>60,78</sup>. Confocal microscope users, developers, and manufacturers must work together to identify and develop methods and standards that will make reproducibility more inherent to all systems, in addition to developing important new tools. Only then can truly quantitative data be achieved routinely throughout the confocal imaging community.



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## Figure Captions

**Figure 1. Quantitative confocal microscopy example.** **a)** Widefield image and **b)** a single confocal image slice of an organoid expressing a GFP nuclear protein (green) and RFP membrane protein (red). **c)** Confocal 3D volume rendering of the organoid. **d)** 3D quantification of the mean nuclear intensities with colour coding from 9000 counts (violet) to 30,000 counts (red). Scale bars = 10µm. Images in a) and b) used with permission from the Journal of Biomolecular Techniques (JBT), ©Association of Biomolecular Resource Facilities, <http://www.abrf.org>.

**Figure 2. Principles of Confocal Microscopy.** **a)** In the Confocal Laser-Scanning Microscope (CLSM), a laser (blue) is focused into a specimen, where fluorescence (green) is excited throughout the entire cone of illumination. Fluorescence generated in the focus (red circle) is imaged through a pinhole onto a detector. Fluorescence from outside the focus, such as the surface (black dashed lines), is blocked by the pinhole. **b)** In the Spinning-Disk Confocal Microscope (SD), a laser (blue) hits a Lens Disk that splits the beam up into 1000 tiny beams, which pass through a matching Pinhole Disk and are focused into the specimen. Fluorescence (green) is 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 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.

**Figure 3. Effect of wavelength on resolution.** MDCK epithelial cells were fixed in ice-cold methanol and stained using a monoclonal anti-tubulin antibody followed by secondary antibodies conjugated to either Alexa Fluor 488 (AF 488) or Alexa Fluor 647 (AF 647). Single optical sections were acquired using a Leica SP8 confocal microscope and a 40x/1.1 NA water immersion objective, with an optical zoom of 4 and the confocal pinhole set at 1 Airy Unit at 580 nm emission. The fact that resolution is inversely proportional to wavelength is well known in theory, but rarely considered in practice. Far-red emitting dyes, such as AF 647, have become increasingly popular in recent years, due to lower autofluorescence in this region of the spectrum and their compatibility with certain super-resolution techniques such as STORM and STED. However, shorter wavelength dyes (blue- or green-emitting) prove significantly better for imaging fine structures at high resolution on confocal microscopes. Scale bar = 10µm.

**Figure 4. Effect of mounting medium on confocal images of fixed cells.** MDCK epithelial cells were fixed in ice-cold methanol and triple-stained by indirect immunolabelling using antibodies against: top row, desmoplakin (DyLight 405); middle row, tubulin (Alexa Fluor 488); bottom row, cytokeratins (Alexa Fluor 647). Triple-labelled coverslips were then mounted using: 1<sup>st</sup> column, Prolong Diamond (uncured); 2<sup>nd</sup> column, Vectashield; 3<sup>rd</sup> column, 100% glycerol; 4<sup>th</sup> column, a second bottle of 100% glycerol. Images (single optical sections) were acquired on a Leica SP8 confocal microscope using a 40x/1.1 water immersion objective and a sequential track for exciting blue, green and far-red emitting dyes. The same tube containing all 3 primary antibodies was used for all 12 coverslips, as well as the same tube containing the combined secondaries. Coverslips mounted using Prolong Diamond or the second bottle of glycerol showed the expected localization of each target antigen. However, Vectashield and the first bottle of glycerol produced bizarre effects. Vectashield preserved the DyLight 405 signal well, but led to a strong reduction in the initial intensity of AF 647, consistent with prior data. However, the AF 488 signal, which is typically compatible with Vectashield, was completely quenched. The experiment was repeated twice more, using a second, brand-new bottle of Vectashield as well as two different AF 488-conjugated secondary antibodies, but the results were consistent. The first bottle of glycerol led to a different surprise in the form of strong nuclear

staining, presumably caused by some contamination. These results are presented not to suggest that the users should avoid the use of Vectashield or glycerol as mountants, but to urge caution in assuming that any unexpected or negative staining results must be the result of the antibody per se: it is important to confirm unexpected results using different mounting media. Scale bar = 10 $\mu$ m.

**Figure 5. Comparing inter-related key instrument performance parameters of different microscopy techniques and configurations.** When choosing between different microscopy techniques it is important to consider how their relative performance attributes, their strengths and weaknesses, make them more or less suitable for a particular experiment. Here we have limited ourselves to 5 key parameters, although your experimental needs may require you to consider others too (e.g. noise). (a) The relative performance of typical WF, SD and CLSMs are compared. It is important to remember that microscopes can be configured differently to optimise for a particular parameter (or combination of parameters), either through the hardware choices or the software settings. (b) Illustrates that the same CLSM instrument can be optimized for either contrast or live-cell imaging, but this comes at the expense of other performance parameters – it is always a trade-off.

**Figure 6. Objective lens comparisons.** a) 20x/0.8NA Plan-Apochromat objective lens corrected for a standard #1.5 (0.17mm) thickness coverslip. The high focusing angle of 53° provides high resolution and sensitivity, but leaves a Full Working Distance (FWD) of just 0.55mm. b) 32x/0.4NA A-Plan objective optimized for a 1mm plastic bottom dish. The FWD is a generous 3mm, but this comes with a reduced resolution (half) and sensitivity (one-quarter) compared to the 20x/0.8NA objective. The built-in phase ring makes it convenient for finding unlabeled cultured cells, but further blocks fluorescence emission. c) 63x/1.4NA oil immersion Plan-Apochromat objective corrected for a #1.5 coverslip. Oil immersion boosts the NA to provide nearly double the resolution and four times the sensitivity of the 20x/0.8NA dry objective, but the tiny FWD requires that you image close to the coverslip.

**Figure 7. The effects of photobleaching during widefield observation.** When preparing for confocal imaging, finding and focusing on your sample in the binocular may result in a significant (but unknown) amount of photobleaching. BPAE cells labeled with DAPI (blue), AF488 phalloidin (green), and MitoTracker Red (red) were sequentially illuminated with an X-Cite 120 widefield fluorescence lamp through the 63x/1.4NA oil immersion objective for 60 s (from red to green to blue channel). a) to d) AF488 image after 0 s, 3 s, 10 s, and 60 s: in just 3 s, the AF488 intensity is half of its initial intensity and by 60 s the fluorescence is completely gone. e) to f) Switching to the 20x/0.8NA dry objective before and after photobleaching (lamp set to 12% power) shows the dramatic decrease in fluorescence in all channels from just 60 s of widefield observation. DAPI has bleached, but photoconversion of DAPI is apparent in the green channel after strong UV illumination. g) Normalized intensity of the 3 fluorophores during 60 s widefield observation at full lamp power. Turn the lamp down to its minimum setting (12% power) to prevent photobleaching. Scale bars = 50 $\mu$ m.

**Figure 8. Configuring confocal detection channels.** a) A screenshot from the Molecular Probes SpectraViewer shows excitation and emission spectra from 4 fluorophores (DAPI, AF 488, AF 568, AF 647). With sequential acquisition, these (or equivalent) fluorophores can be acquired without any crosstalk. b) to d) Screenshots from one of the CLSM configuration wizards, showing b) simultaneous, c) semi-sequential, and d) fully sequential strategies for capturing 3 fluorescence channels. To speed up acquisition, the semi-sequential approach is often recommended by the vendors; but if DAPI is significantly stronger than AF568 in this example, there will be considerable bleedthrough of the

DAPI signal into the AF568 channel. e) Pure DAPI channel from a kidney tissue showing labeled nuclei. f) Nuclei bleedthrough into the green AF488 channel when these 2 channels are imaged simultaneously. g) The pure AF488 is seen when the DAPI laser is switched off – there should be no nuclear signal visible. h) and j) Properly exposed MitoTracker Red staining shown in h) red pseudocolor and j) grayscale with saturation Look-Up Table (LUT). i) and k) Overexposed MitoTracker Red staining shown in i) red pseudocolor and k) grayscale with saturation LUT clearly indicating overexposed (saturated) pixels in red. Scale bars = 10 $\mu$ m.

**Figure 9. Unexpected instrumentation issues cause uncertainty in intensity measurements.** a) Fixed BPAE cells imaged on a CLSM at the maximum field-of-view (Zoom 0.7) using a 20x/1.0NA water immersion objective lens. There is some apparent darkening of the image in the corners. b) Replacing the cells with a uniform plastic fluorescent slide (Chroma), the illumination non-uniformity can be measured (this is called a “Shading Reference” or “Flat-field” image) and potentially corrected. c) Plotting a line profile along the dashed line in b), the extent of the non-uniformity is now more obvious: there is a 60% change in intensity from the corner to the middle of the field at the lowest zoom (zoom 0.7), and still a 40% change at zoom 1. d) Focus drift measured on a new CLSM over several hours, revealing a widespread problem on confocal microscopes: within the first hour of powering on the microscope stand, the built-in focus drifts by more than 1  $\mu$ m every 10 minutes. e) The one hour warmup routine of one model of femtosecond laser – this routine was designed to maximize the power available to the user rather than favouring reproducibility. f) Laser power as a function of time on a heavily used CLSM for the first 2 hours after turning the instrument on. Users (as well as facility staff) were unaware that the most popular laser line (488nm) was fluctuating by about 12% continually. g) The power of the 488nm laser line across 9 CLSMs, as measured at the specimen plane through a 10x objective (with the laser fixed in the center of the field of view rather than scanning). The power varies 50-fold between instruments. Also, bars B1 and B2 show a two-fold change in power after replacing a laser on the system. Scale bars = 50 $\mu$ m.

**Figure 10. Sampling and Statistics.** a) Fluorescence image of eGFP tagged protein localized to adhesion structures. Scale bar = 10  $\mu$ m. b) Adhesion assembly rates were calculated for 10 adhesions from each of 3 cells for a total of 30 in each of 3 independent experiments. Data for each experiment is shown as a box plot. The line=median, open diamond=mean, upper bar= 75th percentile, bottom bar=25th percentile of the distribution. Due to inherent biological variation it is expected that variability between the three experiments is high (SD=40%). c) Box plot of adhesion assembly rates for ten adhesions from each of 9 different cells. Inherent biological variation and variation between adhesion dynamics lead to high variation (SD=45%). d) Box plot and e) bar graph of adhesion assembly rates from 3, 10, 30 or 90 adhesions. Comparison shows the importance of using box plots showing individual data points and statistical information is much more informative than simple bar graphs. The box plot shows that 3 data points cannot clearly represent the entire sample distribution, 10 points begins to show the distribution and 30 points or more is ideal for characterizing the entire distribution. f) A second set of cells was treated with growth factor that increases adhesion assembly rates. Two tailed, unequal variance t-test shows that even with 10 data points the treatment leads to a statistically faster mean adhesion assembly rate with greater than 95% confidence while 30 or 90 data points leads to greater than 99.9% confidence that the mean of the two distributions are not the same.

### **Box 1. The Hazards of Colocalization**

Colocalization analysis can be a powerful tool for assessing the spatial relationship between two probes. It can indicate that two probes are generally near each other, but not that they interact. The accuracy of colocalization (i.e. just how close two molecules are) is limited by the resolution of the imaging system, which ranges from about 200 x 200 x 800 nm for a confocal down to 10s of nanometers for some super-resolution methods. In the confocal microscope, colocalization analysis can therefore at best indicate that two proteins, each a few nm in diameter, are within 200 - 800 nm of each other. Moreover, the choice of labelling technique will also influence the distance between two fluorescent labels. If an indirect antibody labelling approach is used, the fluorochrome can be positioned up to 20 nm from its target epitope. Hence, while colocalization provides an initial hint as to whether proteins could be positioned close enough to interact, other imaging methods (FRET, FCCS, PLA – see the section *Quantitative measurements of dynamics and interactions*) or biochemical means are required for verification. When measuring colocalization, keep in mind the following hazards that can produce false positive and negative results.

#### **You see apparent colocalization where there should be none due to:**

- Cross-reactivity between the two sets of primary and secondary antibodies;
- Binding of your primary antibodies to unexpected additional proteins with similar epitopes;
- Excitation cross-talk between the two fluorophores;
- Emission cross-talk/"bleed-through" between the two fluorophores, particularly if the signal intensities of the two channels are wildly different;
- Use of hardening mountant so that the tissue is squashed in the z-axis, causing the signal from a fluorochrome labelling one cell to appear in the same 3D-voxel as the signal from a different fluorochrome labelling a cell above or beneath;
- Insufficient axial resolution to discriminate between cells overlaying each other due to the pinhole being opened too far;
- Low NA objective leading to poor lateral and axial resolution;
- Spherical aberration caused by refractive index mismatch or wrong coverslip thickness leading to poor axial resolution;
- Insufficient sampling (pixel size too big);
- Inappropriate analysis such as:
  - Failing to exclude background pixels from the analysis;
  - using a pixel-based intensity correlation algorithm in a sample with high background signal in both channels;
  - setting inappropriate thresholds for one or both channels;
  - using pixel-by-pixel colocalization analysis when object colocalization would be more appropriate.

#### **True colocalization within a certain feature is not detected, due to:**

- Poor labeling, often due to incomplete penetration of primary and/or secondary antibodies into the specimen;
- Poor signal-to-noise ratio (SNR) in one or both channels;
- Lateral or axial chromatic aberrations in the objective;
- Misaligned lasers or filters in the microscope;
- A mismatch in brightness between the two channels, such that a simple overlay of the two appears visually almost identical to the single labelled image;
- Inappropriate analysis such as:
  - setting inappropriate thresholds for one or both channels;



- using pixel-by-pixel colocalization analysis when object colocalization would be more appropriate.

**Box 2. Troubleshooting unexpectedly weak fluorescence signal.**

**A. Is it the microscope? Check illumination, configuration, microscope components, detectors:**

1. If there is no signal at all, neither in the binocular nor in confocal mode, check:
  - Are all of the hardware components (main switch/ laser, including key) turned on?
  - Is there a physical shutter inserted in the microscope?
  - Can you see whether the lasers are emitting light?
  - Try restarting the system – a component may not have initialized properly.
2. If the sample appears weak and/or blurry in the binocular, check:
  - Is the fluorescence lamp on and dialed to an appropriate power level? Keep it low to avoid photobleaching, dim the room lights, and allow your eyes to accommodate.
  - Is the objective lens clicked fully into position?
  - Is a polarizer (for DIC) inserted into the microscope? Since lasers are already polarized, it should be removed (keep just the analyzer in place in front of the transmission detector).
  - Is the objective lens clean, and are you using it with the correct immersion medium?
  - Is the spring-loaded front element of the objective lens stuck down? Or is it compressed by hitting the stage insert?
  - Do you have sufficient working distance to focus on the specimen? Oil and water immersion lenses may have only 100µm before the lens physically hits the sample.
  - If the objective has a correction collar, is it set to the correct position?
  - How does it look with a different objective?
  - Could the objective be damaged? (oil inside, scratched) – take it out and check it under a stereo microscope; acquire PSFs using beads/test samples.
3. If the intensity appears normal in the binocular but confocal images are weaker than expected, check:
  - Is the correct configuration setting (filters, beamsplitters etc.) in place for the respective laser wavelengths and detector channels?
  - Is the laser power set at a reasonable level?
  - Is the laser weak? Has it warmed up fully? Is it getting old (dying)?
  - Do you get a similar intensity of signal if you send the emission to a different detector? (i.e., is it a detector problem?)
  - Use a power meter to measure laser power through the 10x objective and compare with previous measurements.

- Try resetting the system – a component in the scanhead may not have initialized properly.

**B. Is it your specimen? Check your reagents, protocol, positive controls etc.**

- Replace your sample with one that you have imaged before and know to be good. Alternatively, try a standard reference slide such as a *Convallaria* section, H&E slide (green or red emission channels only) or Molecular Probes Prepared Slide #1 or #3. Can you get a “good” image with the test slide? Compare the intensity and quality to an image that was previously captured on this microscope (reuse the same settings if possible).
- Is this an old sample? How was it stored? Have you imaged it extensively before so that it is photobleached?
- If you are using tissue, was it fresh or has it been stored for a long time in the freezer?
- If you have imaged a similar sample successfully before, did you change anything in the sample prep (e.g. fixation, coverslip thickness, new/old batch of antibody, mounting medium)?
- If you are doing antibody staining, did your staining procedure work? (positive controls are key) If not, compare different primary antibodies against the same protein, also different secondary antibodies.
- Did your antibodies penetrate throughout the whole thickness of the specimen, or do you need to test different fixation/permeabilization combinations?
- Is your mountant/antifade compatible with the fluorescent probes used?
- If it's a new type of specimen, is it too thick or mounted too far from the coverslip?
- For live cells, did your transfection work? Cell sorting may detect very faint signals (the sum of fluorescence from the entire cell) that are not sufficient for imaging.
- Fluorescent protein (FP) signals may quench when fixed – consider an antibody labeling against the FP with a bright fixed-cell fluorophore.

**Box 3. Troubleshooting blurry confocal images.** a) Fixed cells mounted in glycerol, imaged with a 63x/1.2NA water immersion objective. The cells were grown, fixed, and labeled in a chambered slide. When mounting, using too much mountant left a small gap between the slide and the coverslip, causing refractive index (RI) mismatch aberrations. b) Same slide imaged with a 63x/1.4NA oil immersion objective. Scale bars = 2µm.

For high-NA objectives, the optical properties of the sample, mounting medium, immersion medium, and coverslip must all match the objective specifications to achieve sharp images. If you obtain blurry confocal images, check the following:

- The objective lens may be dirty or damaged (this will also be apparent in the binocular). Follow your vendor or facility instructions for cleaning the objective, usually with lens cleaning solution and lens tissues. Dried oil may take 2 or 3 tries to remove. Remove and inspect for damage: sometimes the seal on the front lens element breaks and oil has leaked inside, requiring a costly repair.

- Most confocals that are configured for the life sciences are equipped with objective lenses that require a #1.5 (0.17 $\mu$ m) coverslip (this is indicated on the lens). Check that you have the right coverslip, and that it is the right thickness.
- Some specialized objective lenses have a correction collar that corrects for the thickness of your coverslip or the RI of your mounting medium. Make sure the correction collar is set appropriately, and try turning it while imaging to sharpen the image (this is difficult with a manual correction collar, but some motorized ones are now available).
- If you have fixed cells and you use a high-NA water objective (eg., 63x/1.2NA water), or if you have live cells and you use an oil objective (eg., 63x/1.4NA oil) the RI mismatch produces aberrations. The effect is not noticeable until you focus 10 $\mu$ m or further beyond the coverslip: if you image thin cells grown right on the coverslip, you will avoid this problem.
- If your objective has a spring-loaded front lens, it is common to accidentally compress the lens elements while trying to focus near the edge of the stage adapter. This will bring the sample into focus, but only with severe aberrations.
- The sample itself might be damaged. Over time, some fluorophores diffuse from the structures they were labeling. Also, tissues may be damaged if the coverslip has slipped, particularly if non-hardening mounting medium is used without sealing the slide.

**Box 4: Troubleshooting jitter and stripes in confocal images.** a) Jitter in a CLSM image of actin filaments, caused by the bi-directional scanning feature (note that the jitter is worse to the left of the image and not apparent on the right side). b) Stripes in an image of a uniform Chroma slide, caused by a bad AOTF driver. Scale bars = 2 $\mu$ m.

Since there are many sources of jitter and stripes in CLSM images, it can be difficult to determine the cause:

- Vibrations are the most obvious cause. Check that the optical table is floating properly, and don't bump the table during acquisition! Also ensure there is no hardware on the table that could cause vibrations (bubbling humidifier bottles, for example)
- If you use bi-directional scanning, it may not be well calibrated. On some CLSMs it is impossible to remove the resulting jitter across the entire field of view.
- A microscope component may be loose (stage, objective lens).
- Usually it is not necessary to work in complete darkness, because the confocal pinhole throws away most background light; but some techniques are more sensitive to stray light, such as two-photon microscopy with NDDs. Try turning the room lights down and dimming or turning off the computer monitor.
- Several hardware problems can result in jitter or stripes, including fluctuations in the laser itself, noise due to AOTF (or AOTF driver). Call your microscope service engineers.

#### **Box 5: Differential Interference Contrast (DIC) complements fluorescence**

With confocal fluorescence microscopy, sometimes the fluorescence labeling is punctate or filamentous such as the Keratin5-GFP labeling in Keratinocytes shown on the left. It may be important to get an overall picture of the cell, for example to assess cell state or confirm cytokinesis. Rather than labeling the cell with additional fluorophores, which may contribute to phototoxicity or otherwise perturb the cell's normal functions, consider adding a transmitted light contrast. Differential Interference Contrast (DIC) can produce sharp images of cell and organelle boundaries as shown on the right, nicely complementing the fluorescence channel (see also Supplementary Movie 1). Scale bar = 10 $\mu$ m.

Unlike phase contrast, which uses a dark ring in the objective to produce contrast (and thereby blocks precious fluorescence photons), the optical elements required for DIC have only a minimal effect on fluorescence intensity. The DIC prism, however, does skew the Point Spread Function (PSF) of your microscope slightly, reducing the resolution, which may not be acceptable for imaging fine details. Some microscope stands have motorized prisms that are automatically inserted for DIC and retracted for fluorescence. Since DIC uses polarized light, it cannot be used with plastic dishes as plastic destroys the polarization. If your microscope is not equipped for DIC, try closing down the Condenser Diaphragm. The resulting "dirty brightfield" image is so-called because it projects blemishes (dust, condensation, etc..) into the image plane that are not visible with proper Koehler-illuminated DIC; but the added contrast may suffice for some experiments.

There are 4 components needed to achieve DIC (2 polarizers and 2 prisms). To add in these DIC elements:

- Adjust the transmitted-light path for Koehler Illumination:
  - focus on the specimen;
  - close down the Field Diaphragm;
  - adjust the condenser focus to bring the edge of the Field Diaphragm into focus;
  - center the Field Diaphragm in the field of view;
  - open the Field Diaphragm back up to just outside the field of view;
  - remove one ocular, and adjust the Condenser (Aperture) Diaphragm to about 2/3 of the full field of view seen with the ocular removed.
- Insert 2 polarizers for viewing DIC in the binocular. One polarizer is subsequently removed for confocal acquisition since the lasers are already polarized.
- Adjust the second polarizer (sometimes called the "analyzer") to make the field of view as dark as possible.
- Insert 2 prisms – one below the objective and one in the condenser turret. Adjust the skew on the objective prism to produce the desired contrast.

**Table 1. Some common objective lenses, key specifications and applications.** There is much more than just the magnification to consider when choosing an objective lens. The Numerical Aperture (NA) determines the resolution and sensitivity of the lens, but also affects the Full Working Distance (FWD).

Table 1

| Magnification / NA | Immersion | Full working Distance (FWD) | Example Application |
|--------------------|-----------|-----------------------------|---------------------|
|--------------------|-----------|-----------------------------|---------------------|

|                                      |                 |         |                                                         |
|--------------------------------------|-----------------|---------|---------------------------------------------------------|
| 20x / 0.8 NA                         | Air (n = 1.0)   | 0.55 mm | Moderate resolution confocal                            |
| 20x / 1.0 NA                         | Water (1.3)     | 2.1 mm  | Intravital confocal / multi-photon                      |
| 32x / 0.4 NA                         | Air (n = 1.0)   | 3.1 mm  | Widefield cell culture                                  |
| 40x / 0.95 NA<br>(correction collar) | Air (n = 1.0)   | 0.25 mm | High-resolution slide scanning                          |
| 63x / 1.2 NA<br>(correction collar)  | Water (n = 1.3) | 0.28 mm | Live-cell confocal<br>(deeper than 10um)                |
| 63x / 1.4 NA                         | Oil (n = 1.518) | 0.19 mm | Fixed-cell confocal; live cell within 10um of coverslip |

## Additional Internet Resources

**Molecular Expressions Optical Microscopy Primer:** <https://micro.magnet.fsu.edu/primer/>

Developed by Michael W. Davidson and his team at The Florida State University, this website hosts a vast amount of knowledge on all aspects of optical microscopy from the physics of light and color and the anatomy of a basic transmitted-light microscope to advanced techniques such as FRET and TIRF.

**iBiology Microscopy Series:** <https://www.ibiology.org/online-biology-courses/microscopy-series/>

Directed by Ron Vale, Nico Stuurman, and Kurt Thorn, this 72-video series (and growing!) starts with the basics of optical microscopy and concludes with some of the latest techniques such as super-resolution microscopy. Their Short Microscopy series of just 14 videos may be more manageable for some.

**iBiology BioImage Analysis Course:** <https://www.ibiology.org/online-biology-courses/bioimage-analysis-course/>

Created by Anne Carpenter (CellProfiler project lead) and Kevin Eliceiri (ImageJ project lead), this series focuses on general steps of image processing as well as introductions to CellProfiler and ImageJ specifically.

## Microscope Vendor websites

Many microscope vendors have educational material on their websites. Some of them license sections from Molecular Expressions, but supplemented with their own microscopes' unique attributes. Others have written their own material from scratch.

**The Confocal Microscopy Listserv:** <https://lists.umn.edu/cgi-bin/wa?A0=confocalmicroscopy>

With an active subscriber base of nearly 4000 participants and an archive that goes back to 1991, the Confocal Listserv is a valuable resource for confocal and related questions. Registration (free) is required.

## Competing Financial Interests

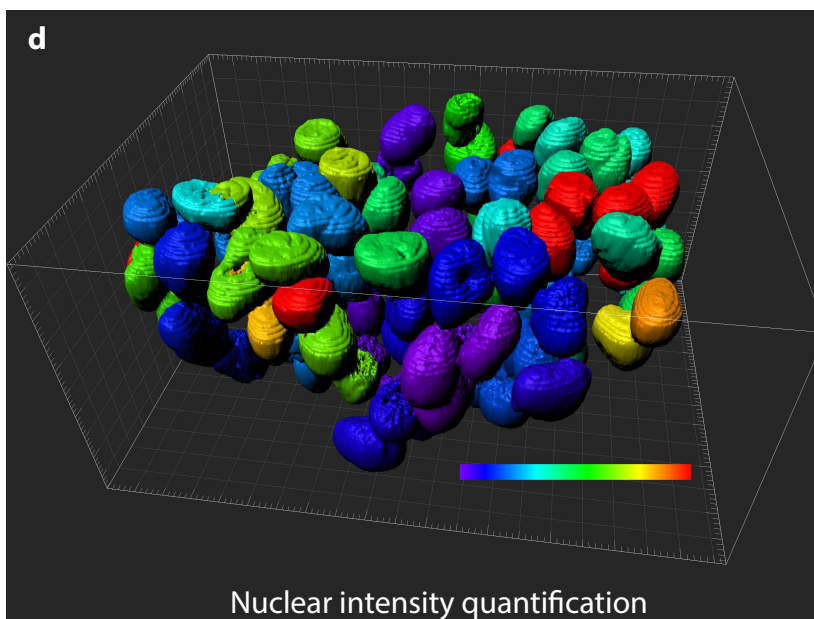
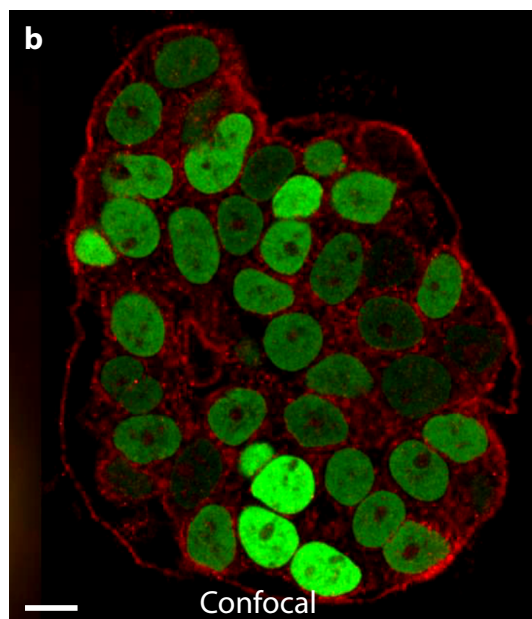
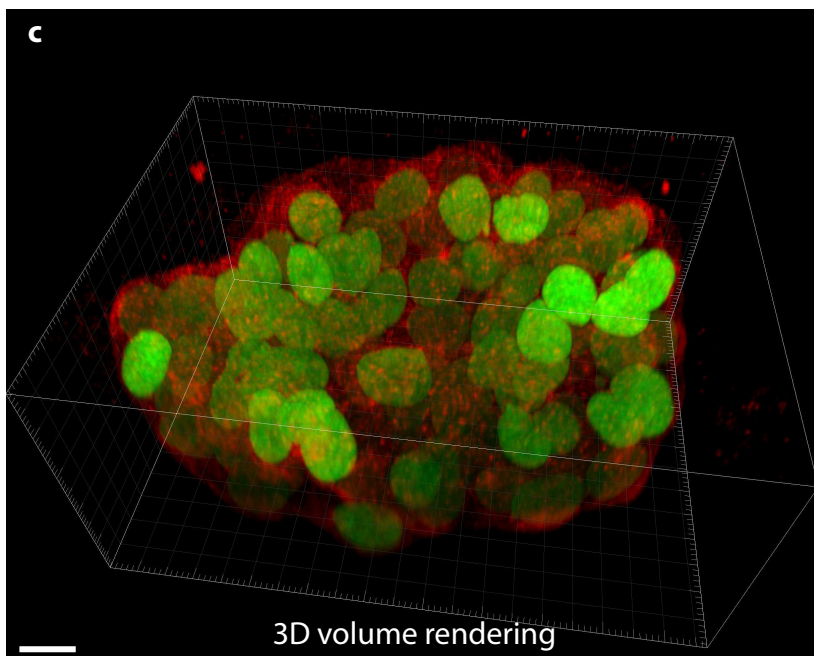
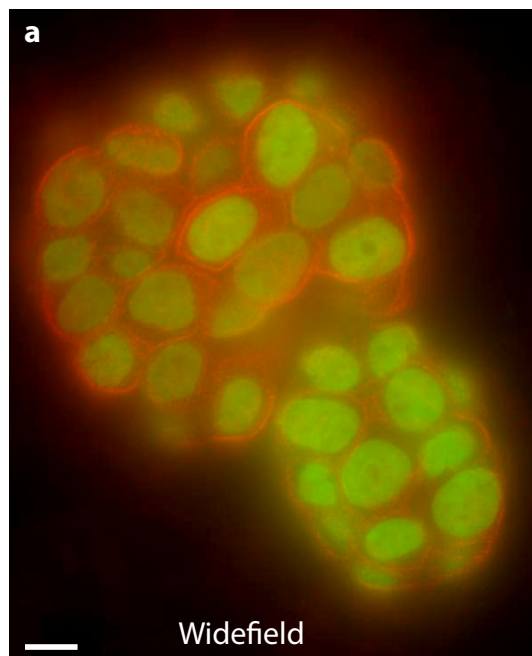
The authors declare that they have no competing financial interests.

## Acknowledgements

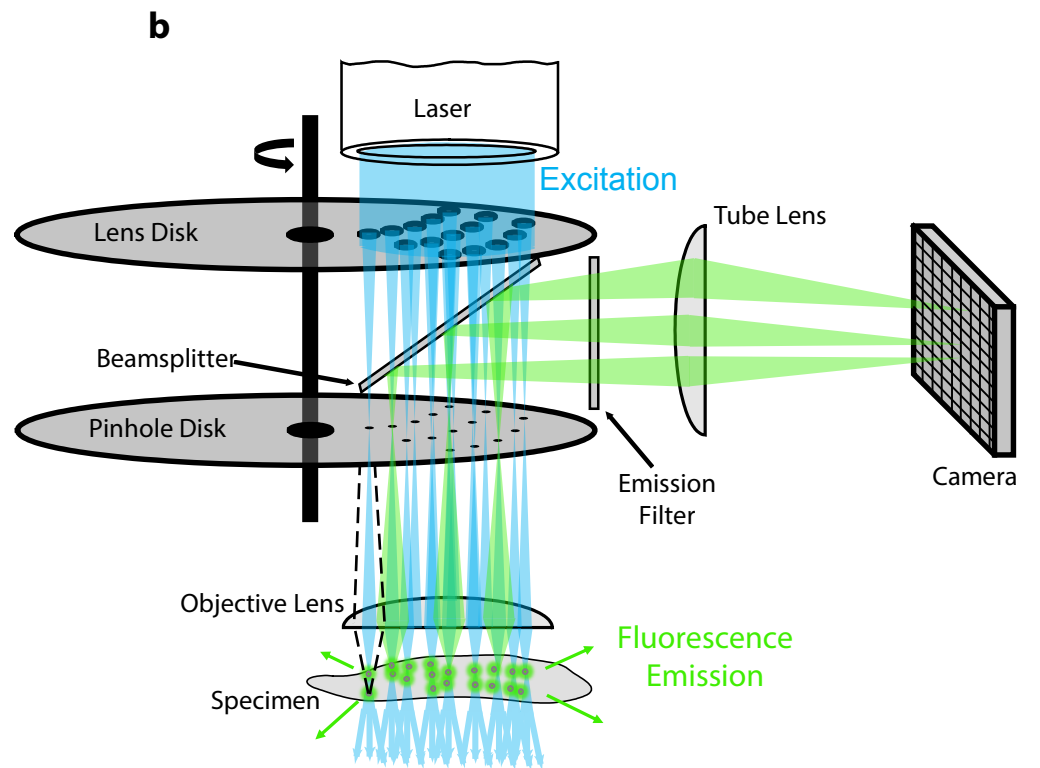
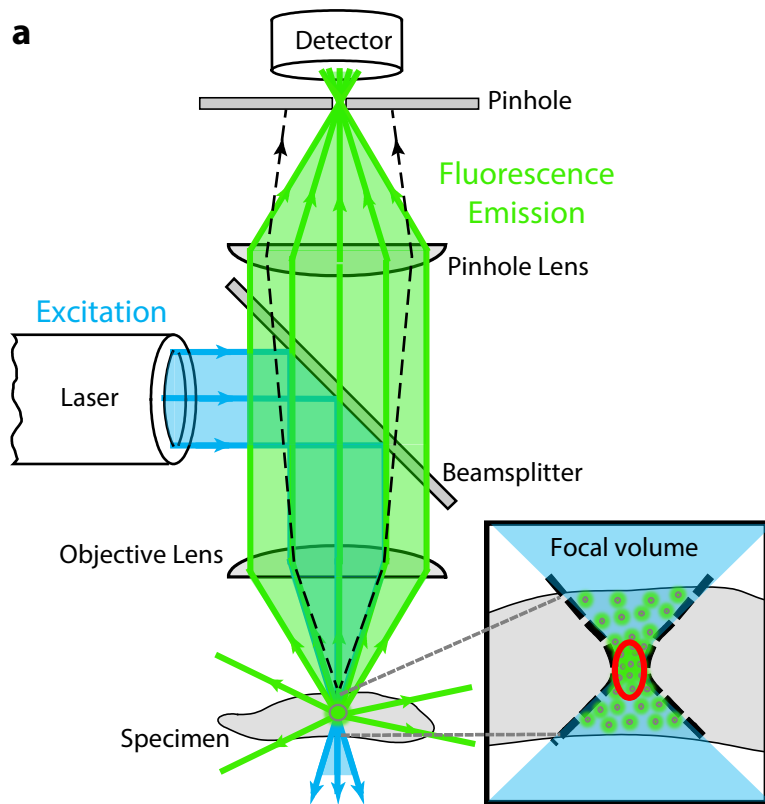
JJ thanks the AOMF staff for helpful discussions, Courtney McIntosh for the images in Supplementary Figure 1, as well as the Princess Margaret Foundation for on-going financial support of the AOMF. GDW thanks A\*STAR and the National Research Foundation's Shared infrastructure Support Grant for continued support of the A\*STAR Microscopy Platform. CMB acknowledges Alex Kiepas, McGill University who collected the adhesion dynamics data for the statistics section of the paper including Figure 10 and the Advanced BioImaging Facility (ABIF) for general support and access to the Discovery spinning disk total internal reflection fluorescence (TIRF) microscope for collecting the adhesion dynamics data. KIA thanks the Francis Crick Institute for their CALM support, and facility colleagues for helpful discussion. AJN thanks the Rockefeller University for its continued support of the Frits and Rita Markus Bio-Imaging Resource Center (BIRC), the Sohn Conference Foundation for funding the Leica SP8 confocal microscope used to generate Figures 3 and 4, and the facility staff and users for stimulating discussions.

## Author Contributions

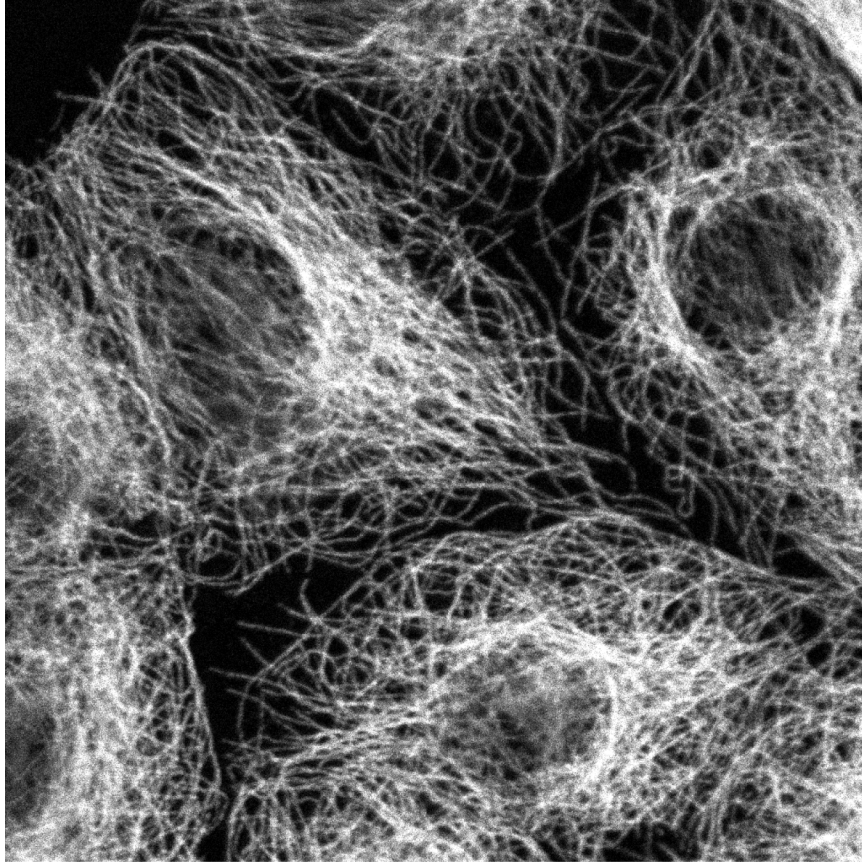
No section of this manuscript was untouched by all 5 authors. JJ drafted the outline, assembled the team, and wrote the *Introduction*, *Removing Bias*, *A Quagmire of Unexpected Instrumentation Issues*, and *Analyzing and Presenting Quantitative Images*. He also generated Figures 1, 2, 6, 7, 8, 9, Table 1, Box 3, Box 4, and Supplementary Figure 1, and contributed to general editing. AJN worked on the sections *Preparing Samples for Quantitative Fluorescence Microscopy* and *Setting Up the Microscope*, contributed extensively to general editing and generated Figures 3, 4, Box1, and Box2. CMB analyzed adhesion dynamics data, generated the statistics figure (Figure 10) and wrote the *Statistics* section of the manuscript. She also contributed to the live cell imaging and sample preparation sections and general editing of the manuscript. GDW worked on the sections *Choosing the Right Microscope* and *Setting up the Microscope*, as well as general editing of the manuscript, and generated Figure 5, the images for Box 5, and Supplementary Movie 1. KIA worked on the sections *Choosing the Right Scope*, *Pilot Projects*, and general editing of the manuscript.



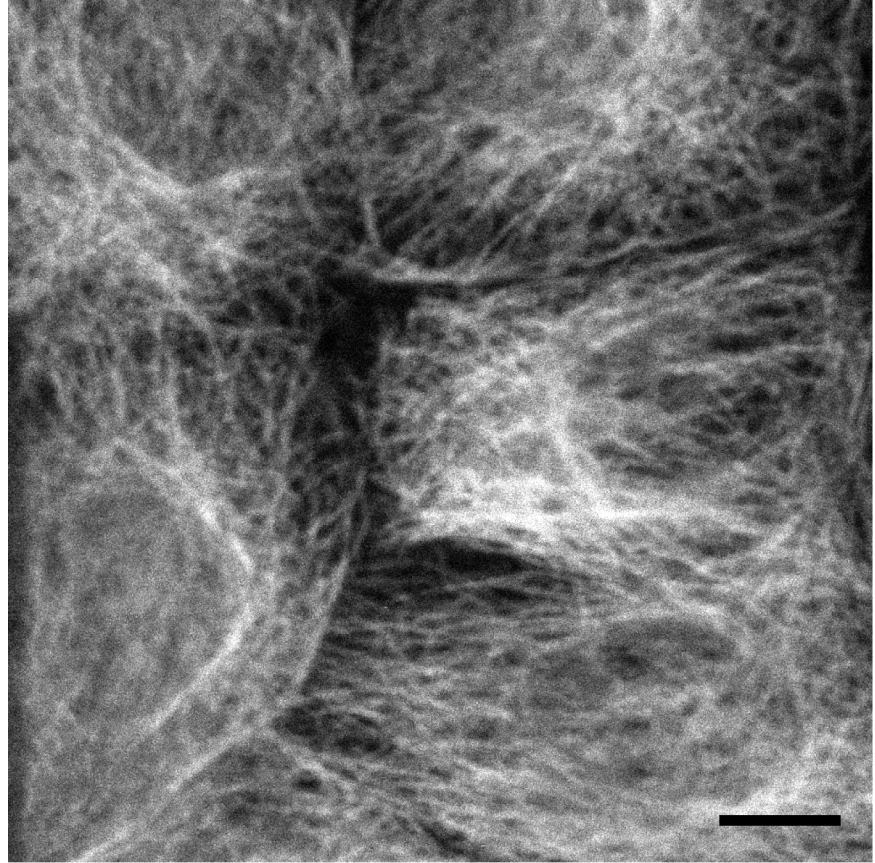




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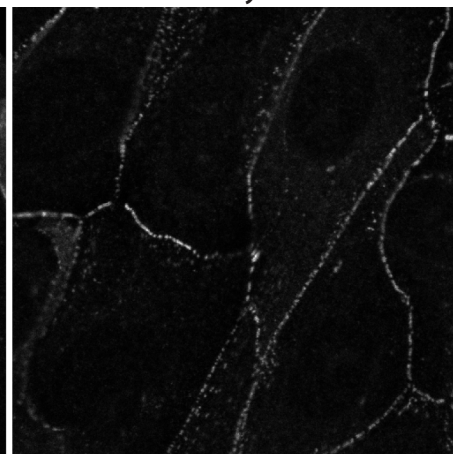
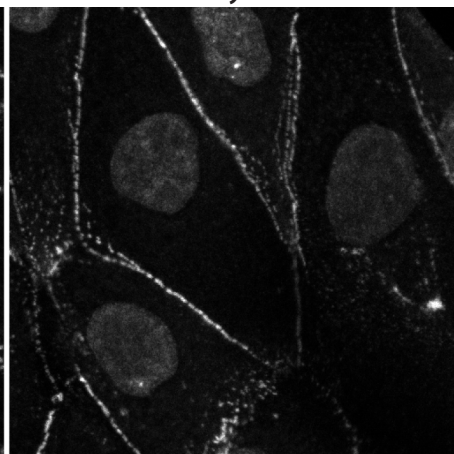
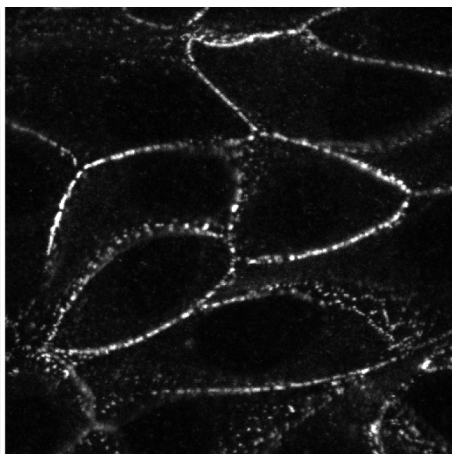
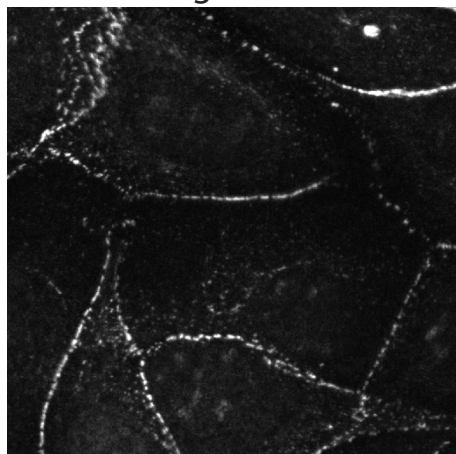
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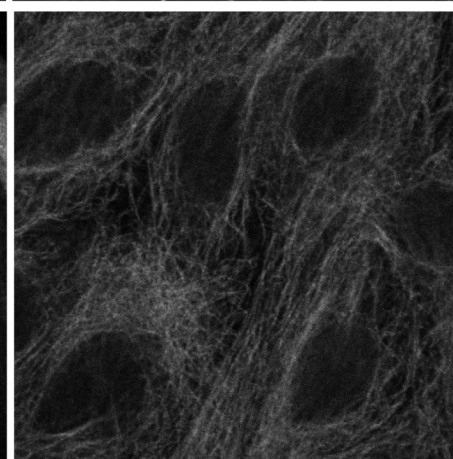
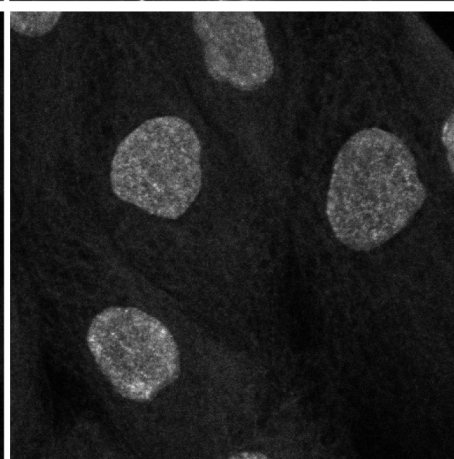
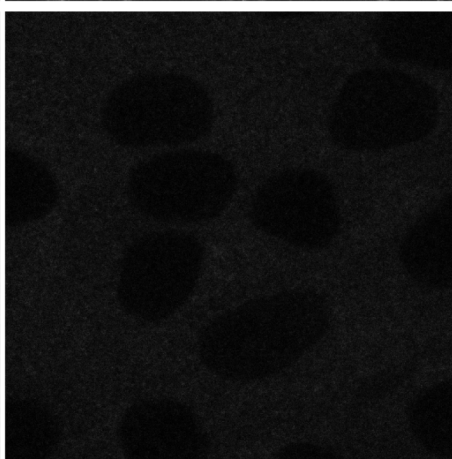
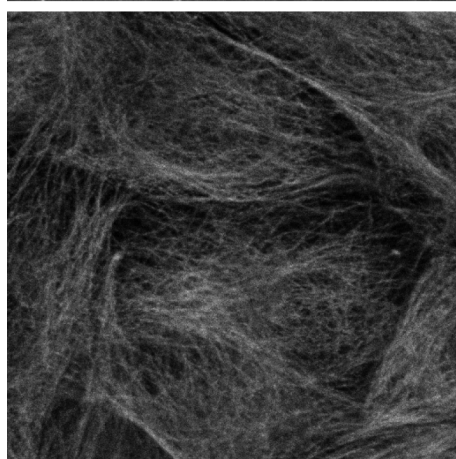
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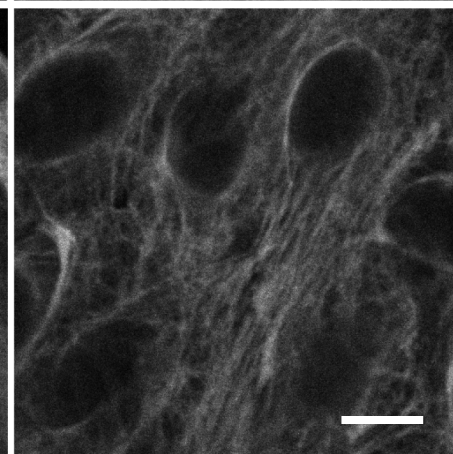
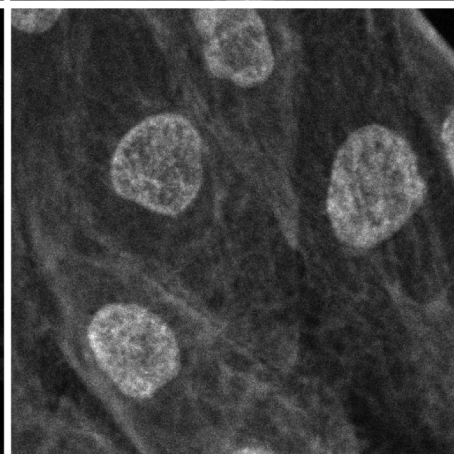
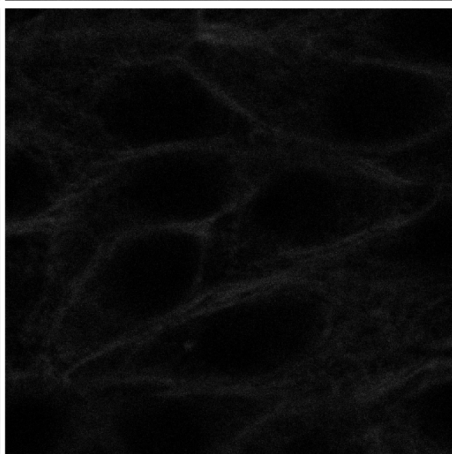
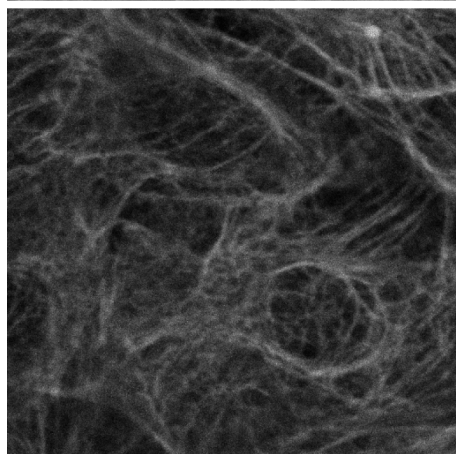
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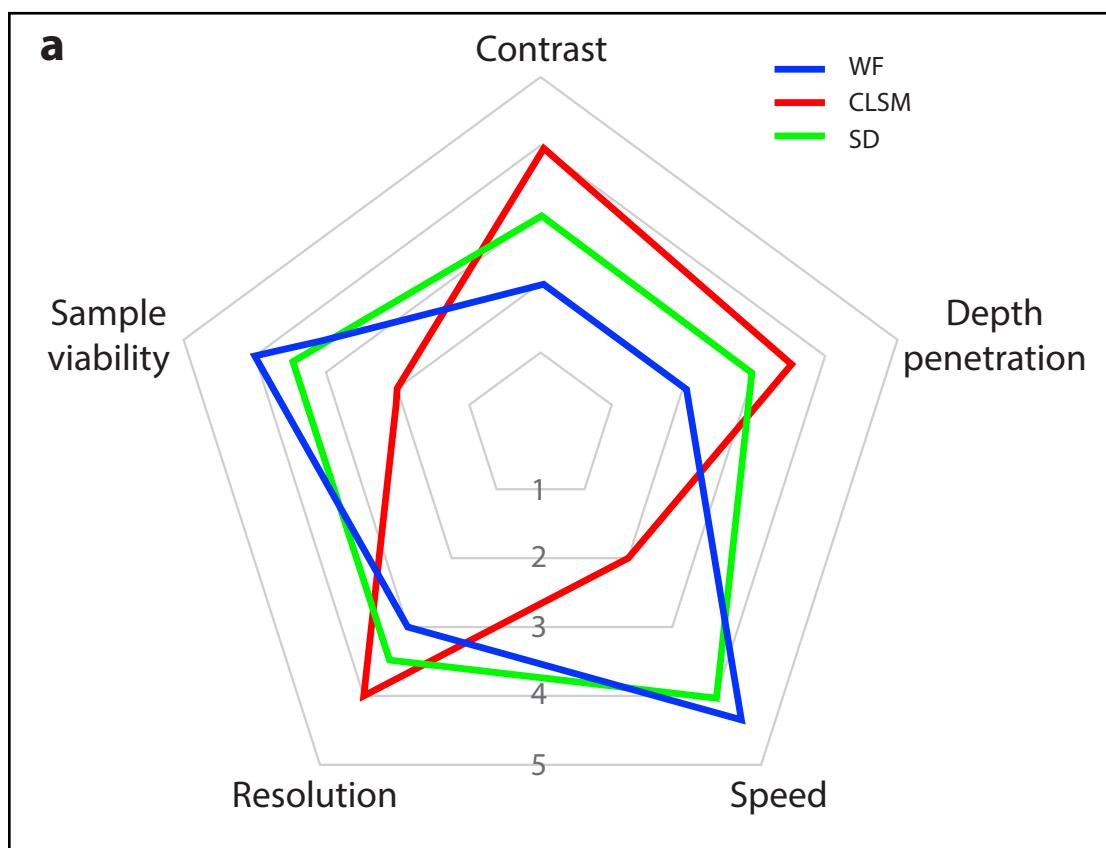


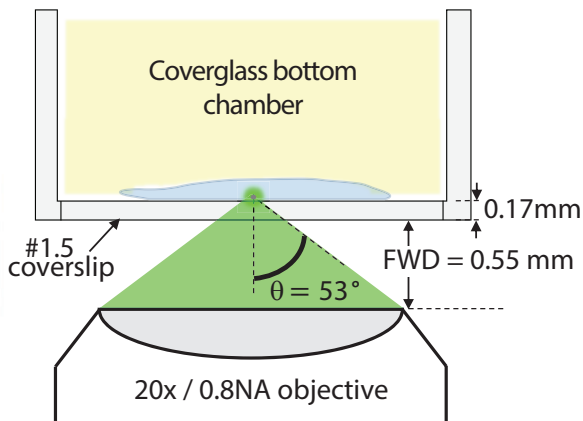
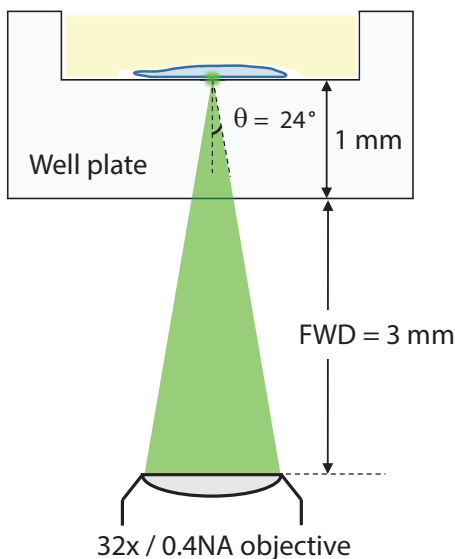
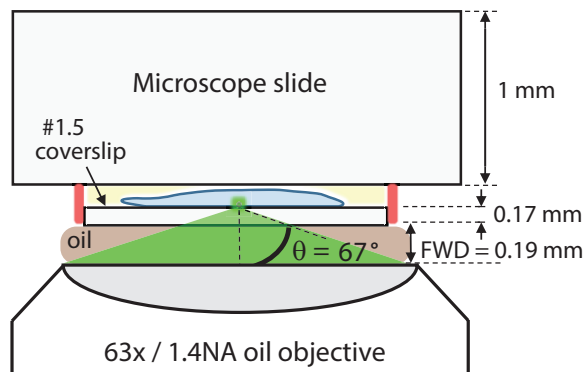
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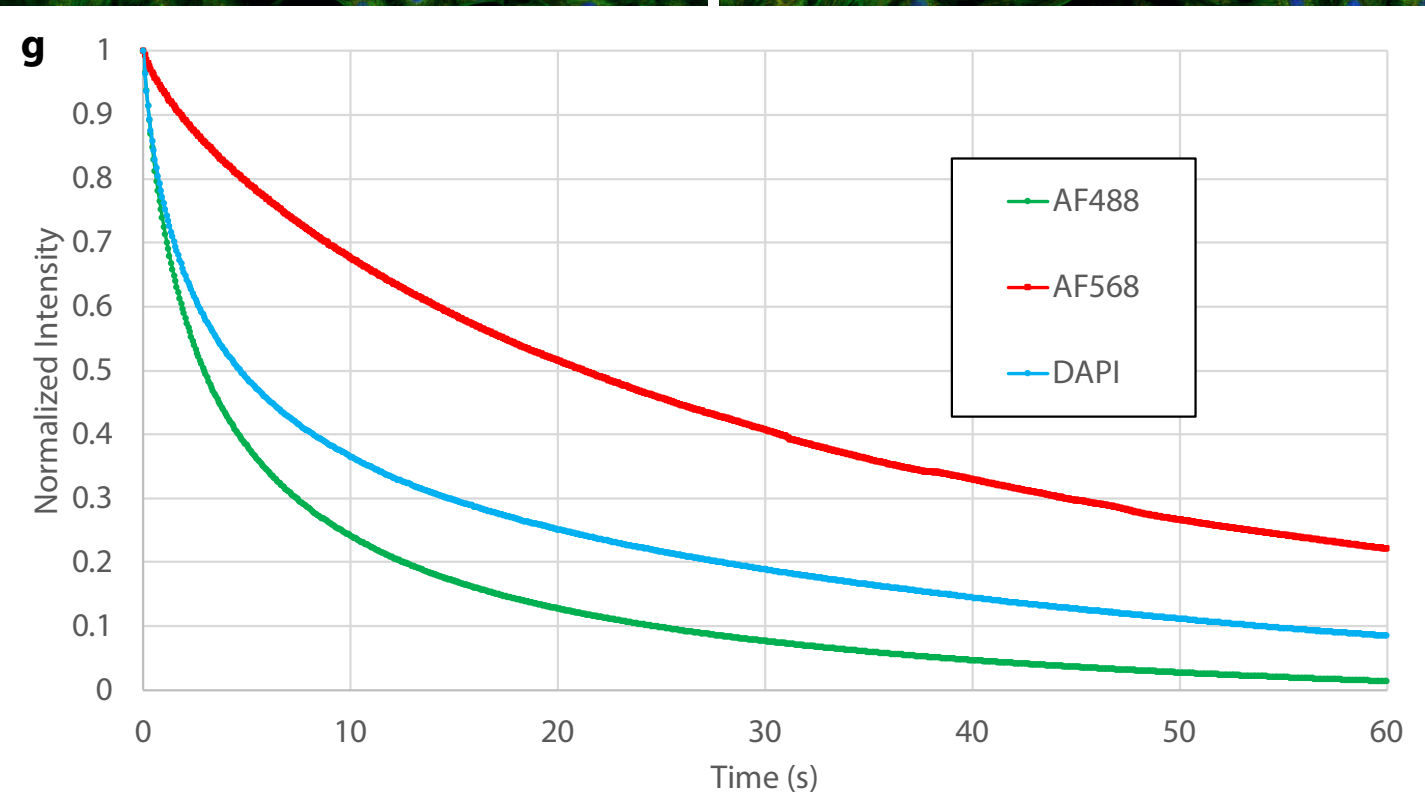
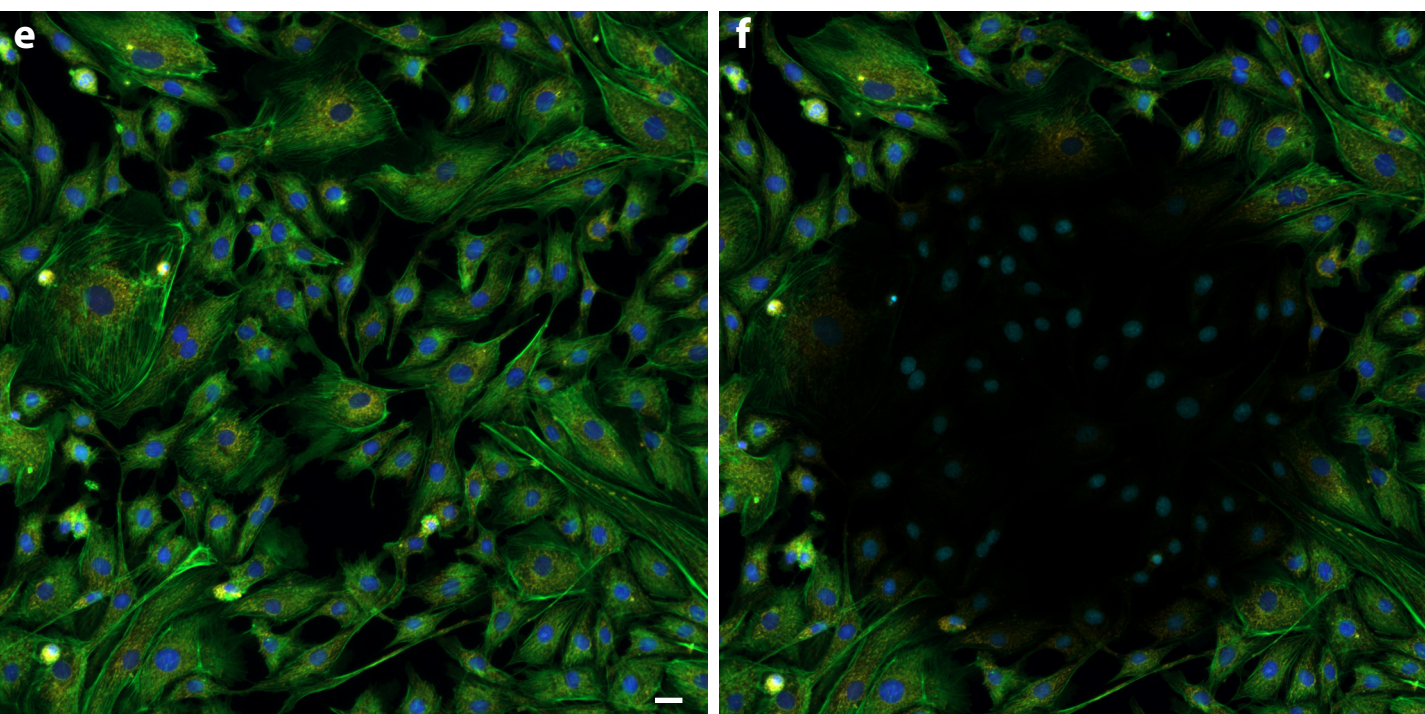
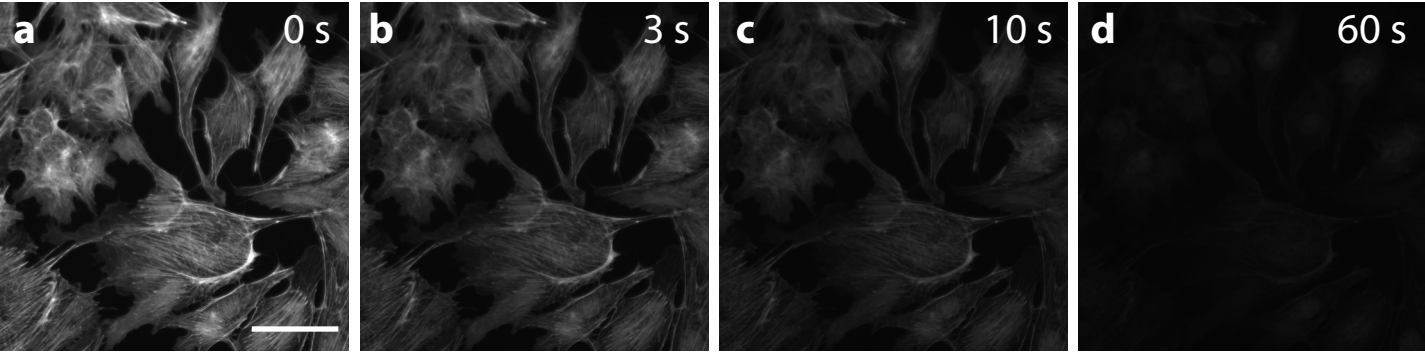
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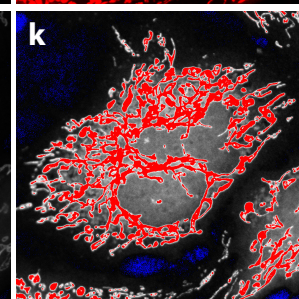
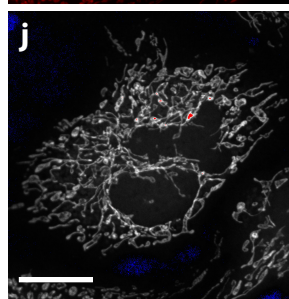
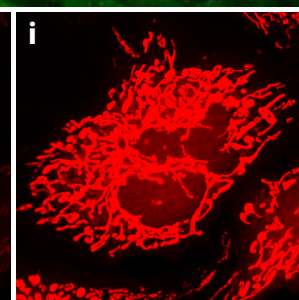
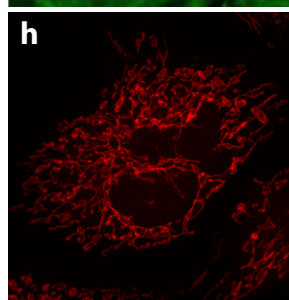
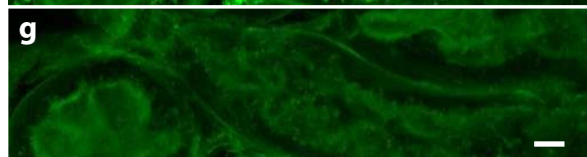
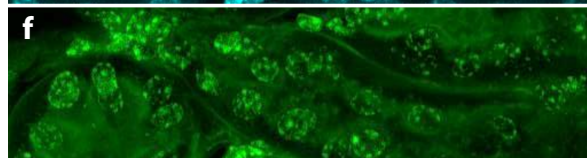
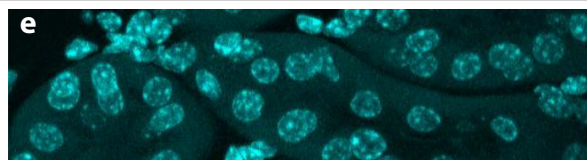
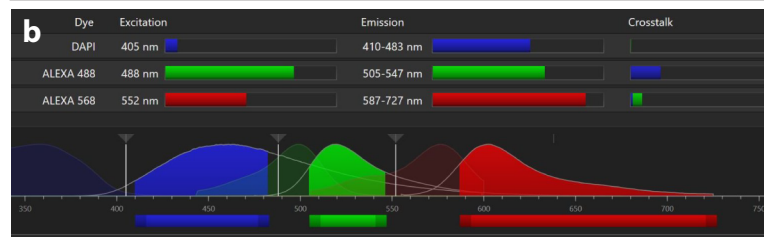
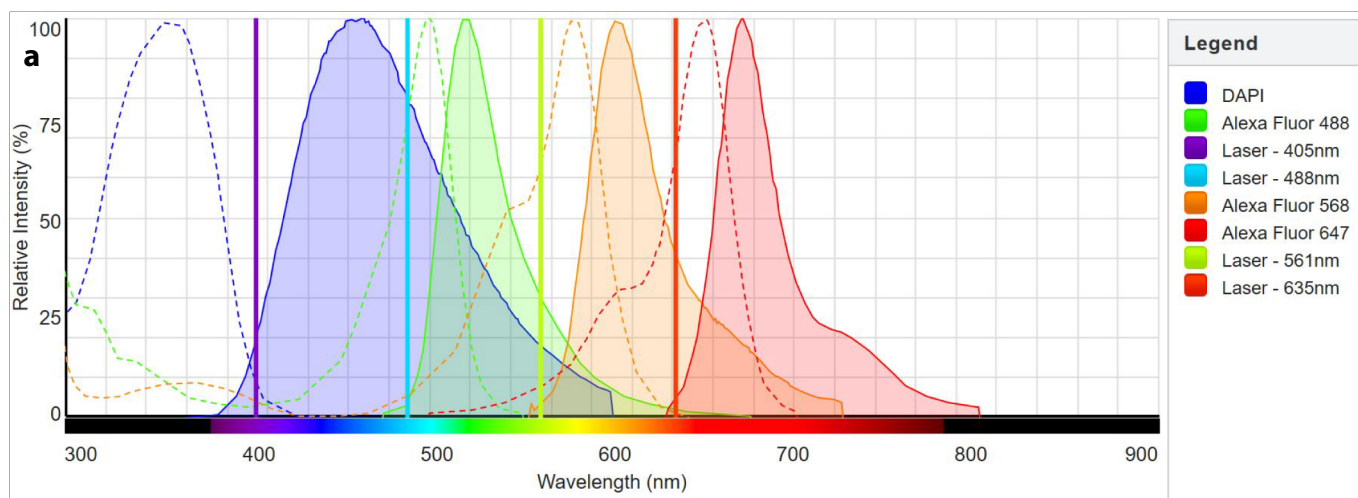


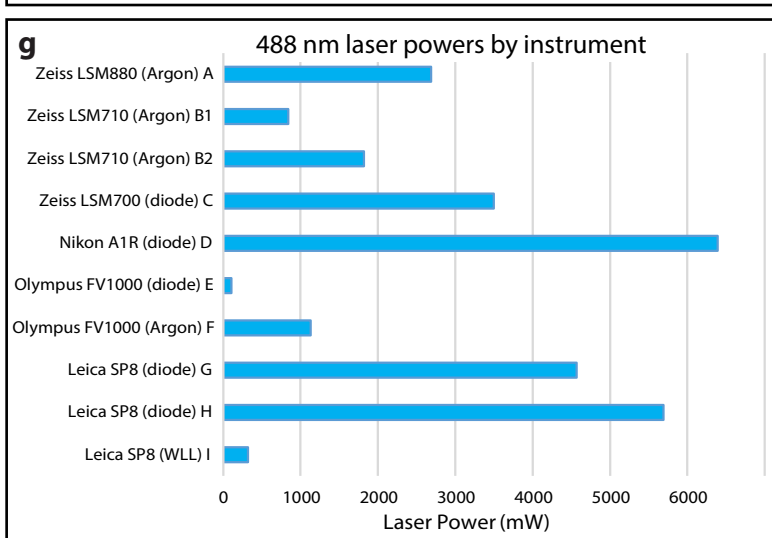
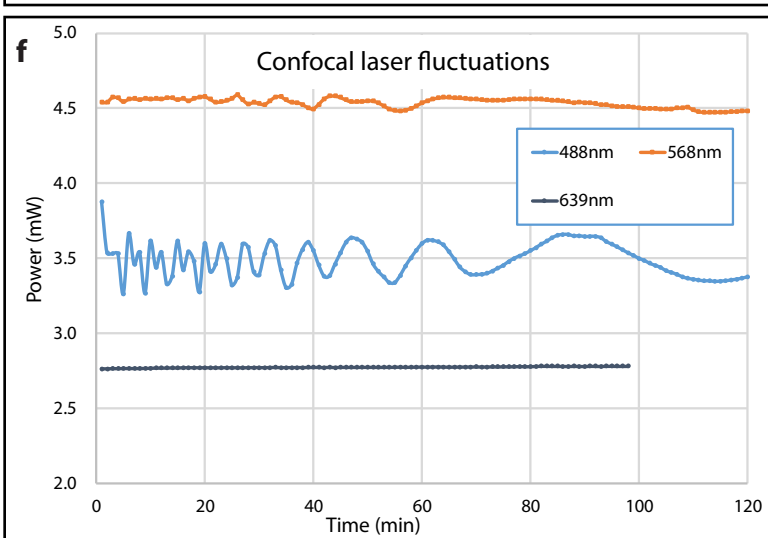
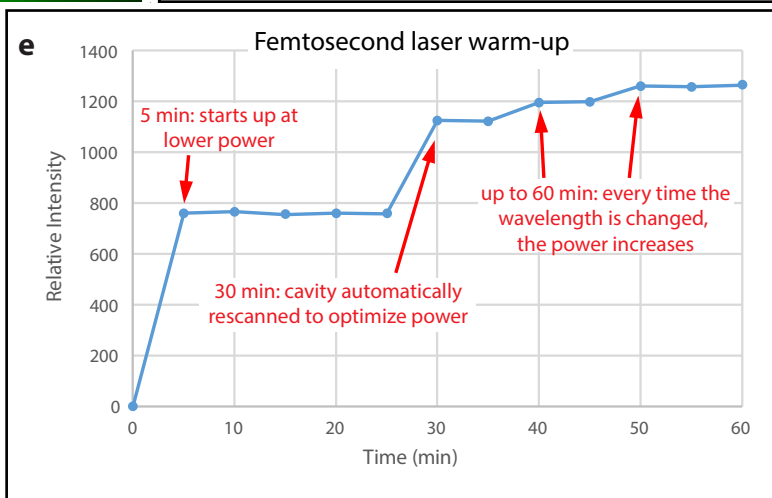
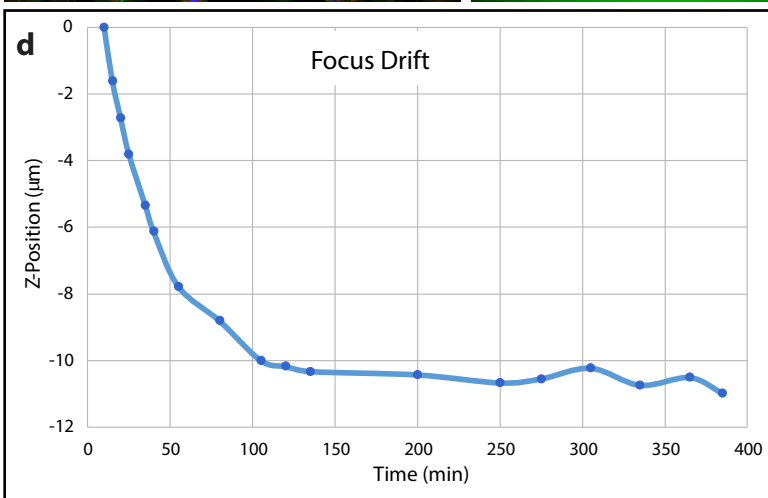
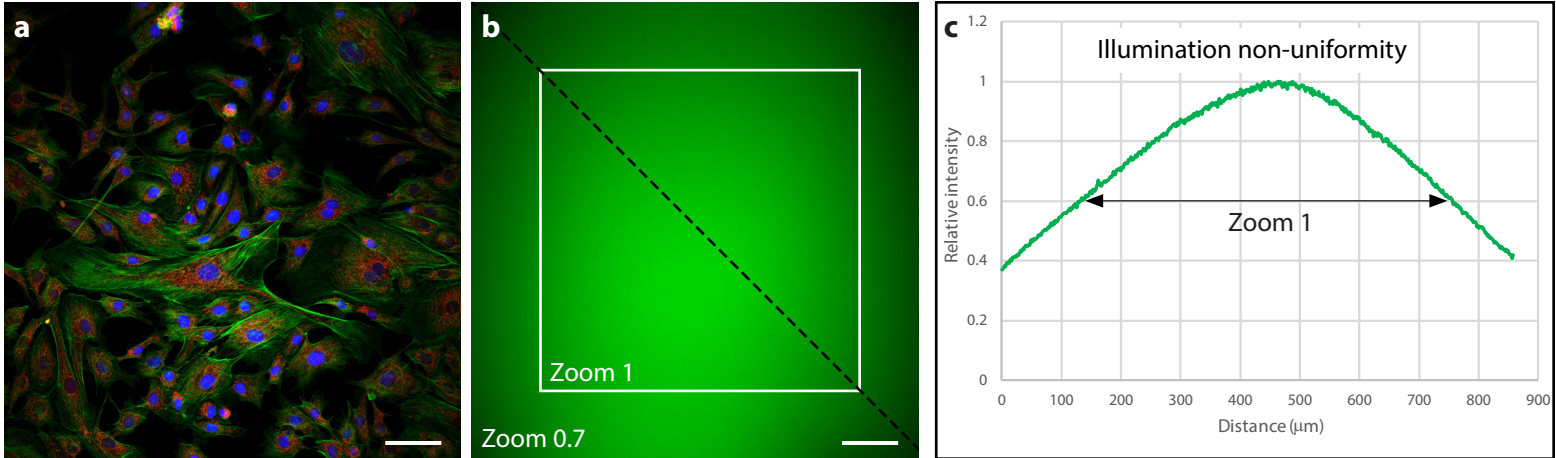


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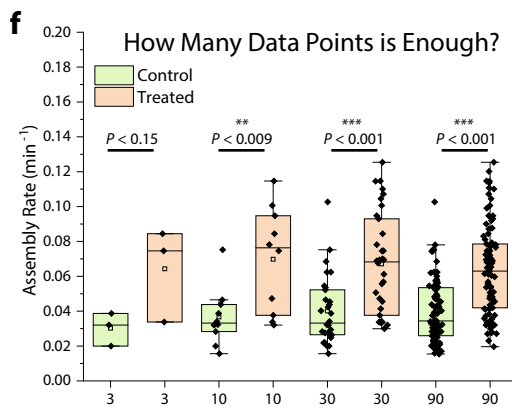
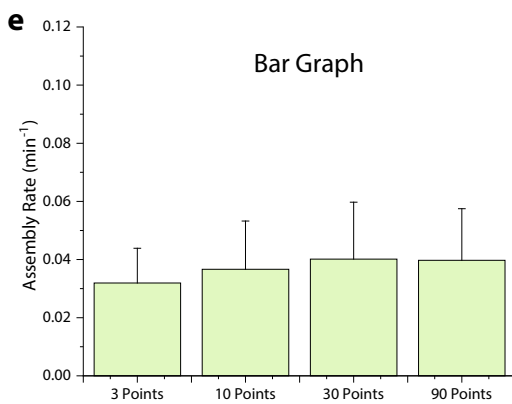
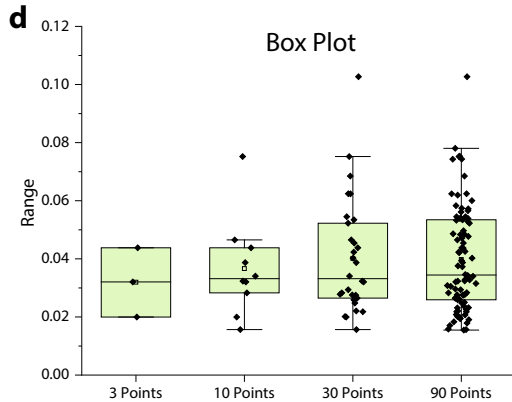
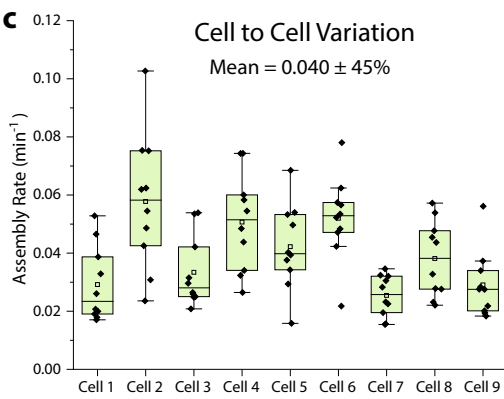
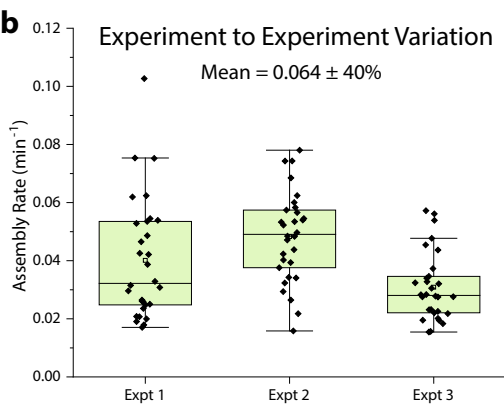
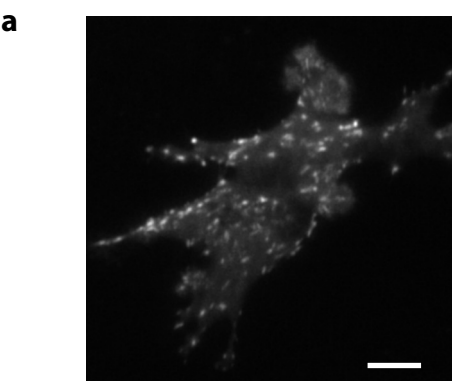


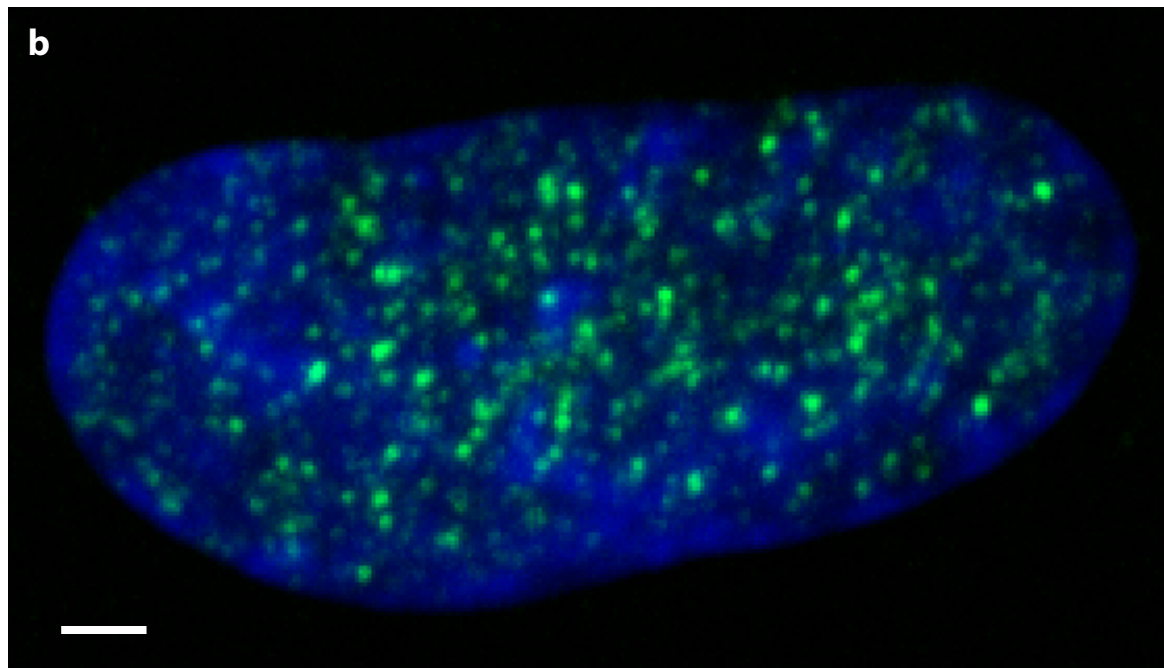
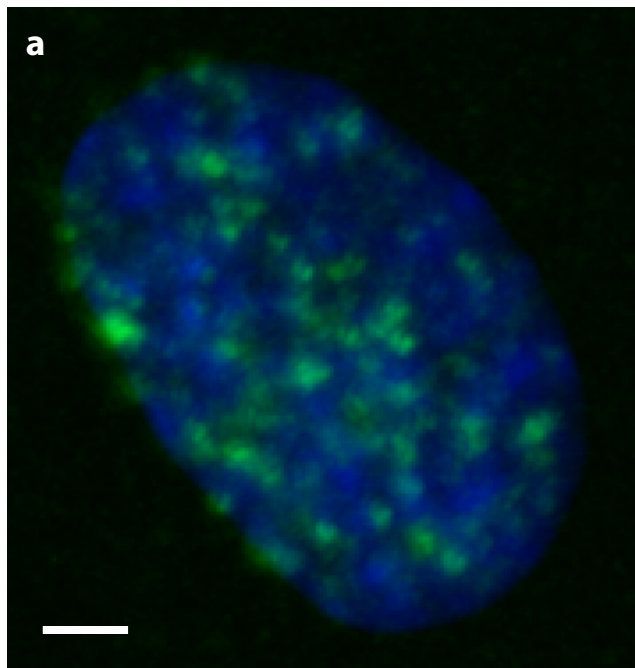


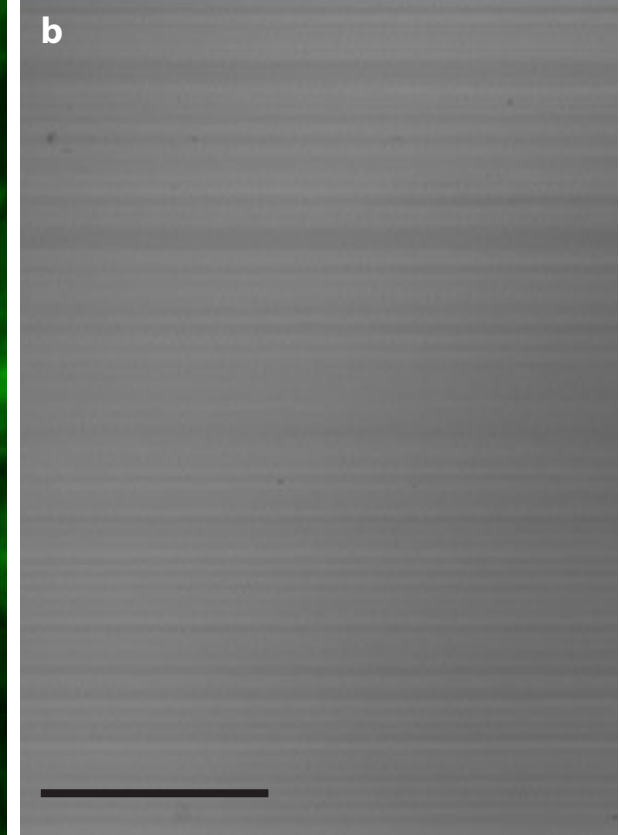
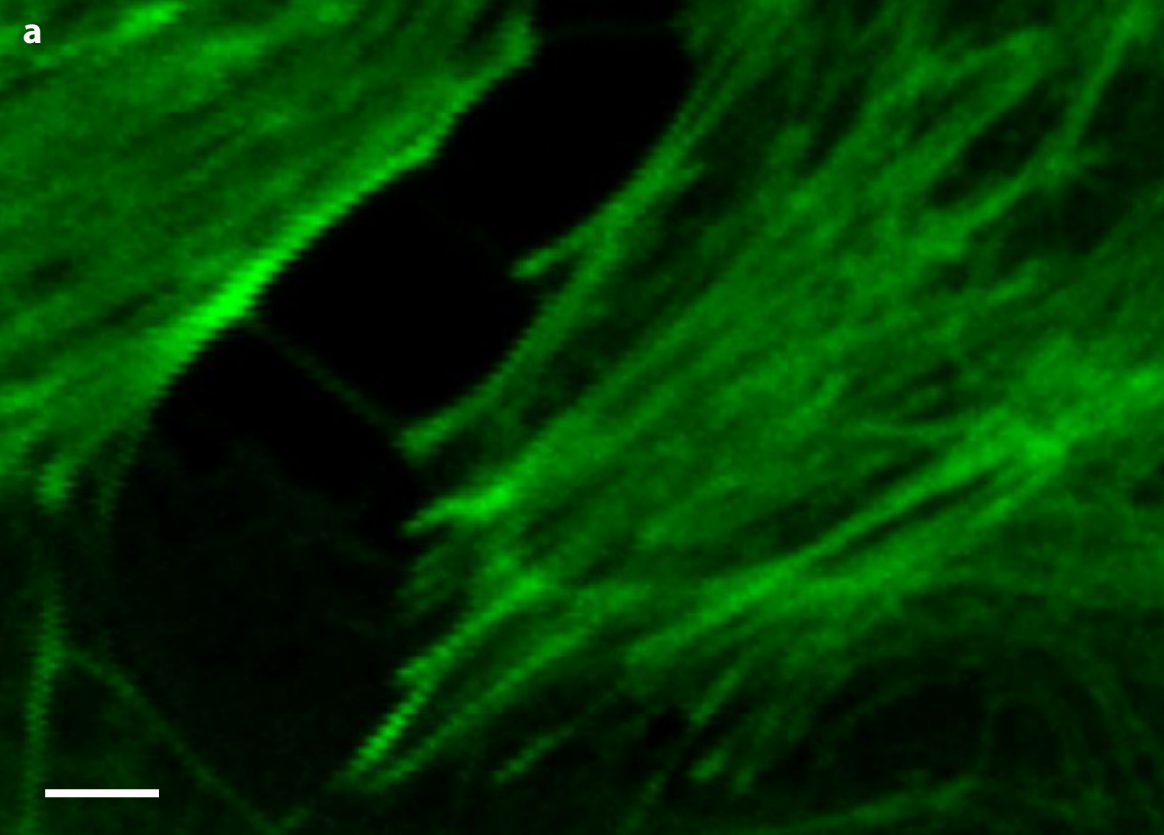












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