Abstract

In recent years, there has been an enormous increase in the publication of spatial and temporal measurements made on fluorescence microscopy digital images. Quantitative fluorescence microscopy is a powerful and important tool in biological research but is also an error-prone technique that requires careful attention to detail. In this chapter, we focus on general concepts that are critical to performing accurate and precise quantitative fluorescence microscopy measurements.
1.1 ACCURATE AND PRECISE QUANTITATION

Designing and implementing a quantitative fluorescence microscopy experiment is most often an iterative and tedious process (Pawley, 2000). Image acquisition settings and analysis tools usually need to be designed and tested multiple times until a reproducible protocol is validated. With each experimental attempt, you are likely to learn something about your specimen, imaging system, or analysis protocol that you can apply to the next round.

Researchers sometimes make the mistake of trying to take the prettiest picture when acquiring microscopy images for quantitation. In quantitative microscopy, it is best to stop thinking about how the image looks and start thinking about the *numbers* associated with the image. A good quantitative fluorescence microscopy experiment is performed with the goal of defining an event or object of interest with numbers, which most often represent fluorescence intensity associated with spatial or temporal measurements. We want our measurements to represent the ground truth (i.e., the reality) of our specimen with high *accuracy* and *precision* (Fig. 1.1; Waters, 2009). Pretty pictures might get you a journal cover, but to obtain reproducible and biologically relevant numbers from live specimens, image quality must be balanced with keeping phototoxicity and photobleaching to a minimum. Therefore, one should identify and use the minimum image quality necessary to satisfy the requirements of the experiment and image analysis protocol, while making every effort to optimize image acquisition to maximize accuracy and precision.

What numbers should you be looking at? A digital image is a grid of pixels, and each pixel has two numbers associated with it: (1) an intensity (aka gray scale) value and (2) a finite-sized area of the specimen that the pixel represents, often called the pixel size (Pawley, 2006). Pixel intensity values are critical. They are not only used as a measure of fluorescence intensity, but they are also used to define objects and to segment the parts of an image to be analyzed. The pixel size determines resolution in the digital image (Stelzer, 1998) and is also important for distance calibration. Other numbers may come into play as well: spacing between images in a *z*-stack or how

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**FIGURE 1.1**

Accuracy and precision. A cartoon of a target and shots demonstrating the difference between (A) imprecision, (B) inaccuracy, and (C) accuracy and precision.
often an image is collected in a time-lapse experiment, for example. In this chapter, we discuss the various numbers associated with digital images and how to use them to design a quantitative fluorescence microscopy experiment. We begin with three critical numbers present in every digital image: signal, background, and noise.

1.2 SIGNAL, BACKGROUND, AND NOISE

A fundamental assumption underlying every scientific experiment is that some ground truth exists that we hope to reveal when making measurements. In quantitative fluorescence microscopy, we measure intensity values of pixels in the digital image in an attempt to reveal ground truths about the localization or quantity of fluorescence in the specimen. For the purpose of understanding the relationships between signal, background, and noise, we will refer to the fluorescence we wish to measure as the signal (Fig. 1.2A). The accuracy and precision of intensity values in a digital image used to measure the signal is degraded, or can even be destroyed, by background and noise (Fig. 1.2B and C; Murray, Appleton, Swedlow, & Waters, 2007; Swedlow, Hu, Andrews, Roos, & Murray, 2002; Waters, 2009). It is therefore critical to understand the sources of background and noise in digital images of
fluorescent specimens, the effect they have on measurements of intensity values, and what can be done about it.

Background adds to the signal of interest, such that the intensity values in the digital image are equal to the signal plus the background (Fig. 1.2B). Background in a digital image of a fluorescent specimen can come from a variety of sources, including ambient light in the microscope room, but the most significant source of diffuse background is usually the specimen itself, for example, fluorescence in the specimen mounting media (e.g., B vitamins, serum, phenol red, and glutaraldehyde-induced autofluorescence) or fluorescence emitted from out-of-focus fluorophores in the specimen (which appears as out-of-focus blur in the image). To quantify a signal, the intensity of background must also be measured and subtracted from the intensity values in the pixels containing the signal of interest (more on background subtraction in Section 1.6.1 and in Chapter 18). It is also important to note that all digital cameras have a certain offset value (Chapter 3); that is, even in complete darkness, pixel intensity values are not zero. Although this “background” is not contributed by specimen fluorescence, it still needs to be removed before quantification.

Before we can understand the full effect of background on measurements of fluorescence intensity, we must also consider noise. Noise is present, to some extent, in every digital image (Chapter 3). Noise causes variation in pixel intensity values from one pixel to the next in each digital image (Fig. 1.2C). Noise causes imprecision in measurements of pixel intensity values and therefore a level of uncertainty in the accuracy of the measurements (Fig. 1.1). To detect the presence of a signal, the signal must be significantly higher than the noise in the digital image. If the signal is within the range of the noise, the signal will be indistinguishable from noise (Fig. 1.3). As the signal increases relative to the noise, measurements of the signal become more precise. The precision of quantitative microscopy measurements is therefore limited (at least) by the signal-to-noise ratio (SNR) of the digital image.

**FIGURE 1.3**
Lost in the noise. (A) A high SNR ratio image of fluorescence beads of two different intensities. Both the bright bead in the center and the surrounding weak intensity beads are visible. (B) Noise was added to the image in shown in (A) using image processing software. The weaker intensity beads are no longer visible, due to the decrease in SNR.
There are multiple sources of noise in fluorescence digital images. We briefly review the most common sources of noise here; they are discussed in more detail in Chapter 3. Counting stochastic quantum events, such as the arrival of emitted photons at the digital camera’s detector, is fundamentally limited by Poisson counting statistics. Poisson noise (aka shot noise) is therefore always present in digital images. If you were to make repeated intensity measurements of the same ideal, unchanging specimen, then you would find that the set of measurements would not be identical, but would instead have a Poisson distribution. Poisson noise results in a standard deviation in the number of counted photons that is equal to the square root of the total number of detected photons. Note that this formula applies to the number of photons detected, not the arbitrary intensity values reported by detectors; Chapter 3 explains how to convert intensity values to photons. Digital images are further degraded by various sources of noise generated by the detector (Chapter 3). These different sources of noise are summed (as the square root of the sum of squares) in the final digital image. The total noise in the digital image defines a minimum expected variance in measurements of intensity values. Differences in measurements that lie within this expected variance due to noise thus cannot be attributed to the specimen.

With an understanding of noise, we can now gain a full appreciation of the detrimental effect of background fluorescence on quantitation of signal intensity. The presence of background decreases the image SNR because Poisson noise is equal to the square root of all detected photons—signal and background. Noise is not a constant and therefore cannot be simply subtracted from the image. While background can be (and must be) carefully measured and subtracted from an image, the Poisson noise resulting from background photons remains and decreases the precision of your measurements.

In addition to degrading the SNR, background in a fluorescence image also effectively reduces the detector capacity. Charge-coupled device (CCD) and scientific complementary metal-oxide-semiconductor (sCMOS) cameras, for example, have a limited capacity to collect photons. If capacity is reached for a given pixel, this pixel will be saturated in the digital image (Chapter 3). Since background photons use up the detector capacity, this limits the total number of photons that can be collected before the detector saturates. Saturation destroys the linear relationship between the number of photons arriving at the detector and the intensity values in the image and therefore must be avoided in quantitative microscopy experiments.

While noise cannot be subtracted from the image, if multiple images of the same unchanging field of view are collected and averaged together (called frame averaging), the noise can be averaged out. Frame averaging can be very useful when imaging fixed specimens but is usually impractical for quantitative imaging of live fluorescent specimens (Chapter 5) that are both dynamic and susceptible to phototoxicity and photobleaching. For quantitative fluorescence imaging, image noise should be reduced as much as possible through optimization of detector and acquisition settings (Chapters 3 and 5).

How should you use this knowledge to improve your quantitative microscopy experiments? Image acquisition software packages used for microscopy applications
have multiple tools to look at the intensity values within the image: pixel intensity histograms, and image or region of interest intensity value statistics (i.e., mean intensity, standard deviation, and min/max intensity). Find these tools, and use them routinely. Looking only at the image displayed on the computer screen can be extremely deceptive because all imaging software packages map the acquired image to $2^8$ (256) gray levels for display (Cox, 2006), while images acquired for quantitation usually have $2^{12}$ (4096) or $2^{14}$ (16384) gray levels. This mapping can be scaled in various ways, which greatly influences how an image looks on the computer screen (Fig. 1.4).

When assessing image quality, look at the intensity values in a region in the background where there is no specimen. How high is your background compared with the camera offset? Is there anything you can do to your specimen to reduce fluorescent background? Compare the background values to the area of the specimen you intend to measure. How many intensity values above background is your signal? Always keep specimen health in mind. If your analysis looks good but your cells look sickly after acquisition, lower illumination intensity or exposure time while monitoring the intensity of the signal of interest above background (Chapter 5). As you go back and forth between acquiring and analyzing images, pay attention to how the SNR and background in the images affects your results.
1.3 OPTICAL RESOLUTION: THE POINT SPREAD FUNCTION

Resolution is the ability to distinguish objects that are separate in the specimen as separate from one another in the image of the specimen. The point spread function (PSF) describes how diffraction of light in the microscope limits resolution and is described in detail in Chapter 10 (Hiraoka, Sedat, & Agard, 1990; Inoué, 1989; Inoué & Spring, 1997). The equations for resolution of the light microscope assume that you are imaging an ideal object that is directly attached to the coverslip and does not scatter or refract light (Chapter 10; Hell, Reiner, Cremer, & Stelzer, 2011), and do not account for aberrations that may be introduced by the optics in the microscope (Chapter 2; Arimoto & Murray, 2004) or limited SNR in the digital image (Chapter 3; Stelzer, 1998). These ideal conditions are almost never met in reality, making it difficult to achieve the theoretical resolution limit. Each lens is different, so empirically measuring the PSF is the best way to determine the resolution limit of your microscope optics (Chapter 10; Cole, Jinadasa, & Brown, 2011; Hiraoka et al., 1990). DNA origami can also be used to make test specimens for measuring resolution, as described in Chapter 25 (Schmied et al., 2012). Methods of correcting for aberrations induced by the specimen are worth considering as well (Fuller & Straight, 2012; Joglekar, Salmon, & Bloom, 2008).

1.4 CHOICE OF IMAGING MODALITY

Your choice of fluorescence imaging modality (e.g., wide-field fluorescence, spinning disk confocal, point scanning confocal, or TIRF) will affect your quantitative measurements. There is no “best” modality for quantifying fluorescent specimens; instead, the most appropriate choice depends on your specimen and what you are trying to measure.

The purpose of confocal microscopy is to reduce out-of-focus fluorescence in the image of your specimen (Chapters 7 and 9; Conchello & Lichtman, 2005). A common misconception is that confocal microscopy provides higher resolution than wide-field fluorescence microscopy (Cox & Sheppard, 2004). Increasing resolution with a confocal microscope is possible but requires setting the pinhole size to be much smaller than the diameter of the PSF (i.e., much smaller than is necessary to reduce out-of-focus fluorescence). Closing the pinhole to the extent that will (in theory) increase resolution in the image is impractical with most biological specimens, since it also severely limits the number of photons collected from the focal plane and therefore reduces the image SNR. One should not think of a confocal as a method of increasing resolution as compared to wide-field fluorescence microscopy, but instead as a method of getting closer to the theoretical resolution limit when imaging specimens that have significant out-of-focus fluorescence. As explained early in this chapter, background fluorescence reduces the image SNR, and sufficient SNR is necessary to achieve theoretical resolution (Stelzer, 1998). Therefore, in quantitative microscopy, the best reason to use a confocal microscope is to reduce out-of-focus fluorescence in order to increase the image SNR.
What is the harm in going straight to a confocal? Point scanning confocal microscopes are $\sim 200 \times$ less efficient than wide-field microscopes at collecting and detecting fluorescence from your specimen. Spinning disk confocal microscopes are far more light efficient than point scanning confocal microscopes but still $\sim 2\text{–}4 \times$ less efficient than wide-field microscopes. Therefore, imaging specimens with a confocal microscope that do not have significant out-of-focus fluorescence will result in lower SNR images, assuming the same illumination intensity and duration are used (Murray et al., 2007).

Total internal reflection fluorescence (TIRF) microscopy uses oblique illumination to generate an evanescent field at the interface between the coverslip and a lower refractive index specimen (e.g., cells in tissue culture media; Chapter 12, Axelrod, 2001). The evanescent field decreases in power quickly with distance from the coverslip, such that only fluorophores that reside within $\sim 100 \text{ nm}$ of the coverslip surface are excited and emit photons. TIRF can provide a $\sim 6\text{–}7 \times$ thinner optical section compared with confocal, leading to a dramatic reduction in background fluorescence and increase in axial resolution, with the critical caveat that TIRF is only useful for imaging the part of the specimen that is within $\sim 100 \text{ nm}$ of the coverslip. For specimens that do reside within the evanescent field (focal adhesions, membrane proteins, endo/exocytosis, in vitro assays, etc.), TIRF is an excellent option. A downside of TIRF for quantitative measurements of fluorescence intensity is the typically highly uneven illumination (due to both the Gaussian laser profile and interference patterns generated by refraction of coherent laser light on dust particles and filter surfaces). With care, a flat-field correction (explained in detail in Section 1.6.2) can be used to correct for uneven illumination in TIRF. Recent approaches to greatly reduce interference patterns include Ring-TIRF (Applied Precision) in which the entire periphery of the back aperture of the objective is illuminated.

There are a wide range of additional fluorescence imaging modalities that may be used for quantitative microscopy, many of which are discussed in this volume: multiphoton (Chapter 8), deconvolution (Chapter 10), light sheet microscopy (Chapter 11), scanning angle interference microscopy (Chapter 13), and superresolution techniques (Chapters 14–17) to name a few. Each of these modalities comes with their own advantages, disadvantages, and requirements when used for quantitative imaging.

### 1.5 SAMPLING: SPATIAL AND TEMPORAL

Sampling is collecting a subset of information about the specimen that is then used to represent the whole (Fig. 1.5). We sample our specimen both spatially and temporally in the course of a live quantitative microscopy experiment. In time-lapse experiments, we sample the dynamics of our specimen over time by collecting images at regular, discrete time points (Fig. 1.5 A–D). We sample the optical image created by the microscope with a limited number of finite-sized pixels to generate a digital
FIGURE 1.5 Sampling. During a typical time-lapse imaging experiment, images are collected at discrete time points with an interval of time between each image. (A) An object of interest is changing in intensity and/or location over time. If an image is collected at the time points indicated by arrows, the (B) analysis of the location/intensity of the object would not accurately represent the changes over time. (C) Increasing the rate of sampling (arrows) results in more accurate analysis (D). (E–F) A simulated image with a small (E) and large (F) pixel size, illustrating aliasing and loss of information as a result of spatial undersampling.
image (Fig. 1.5E and F). In addition, we may sample our specimen in 3D by collecting a z-series with a constant spacing between 2D images. In all of these dimensions, it is essential to sample with a sufficiently high frequency so that the collected digital image or image sequence can adequately represent the spatial and temporal information that is to be analyzed. Undersampling can result in loss of information (Fig. 1.5B and F), while oversampling can result in unnecessary specimen damage without providing additional data. Ideal sampling is rarely possible when imaging live, dynamic, photosensitive specimens. We may be limited, for example, by the rate of photobleaching or by how fast the motorized components of the imaging system can move.

1.5.1 2D SAMPLING

As discussed in the preceding text, resolution of the optical image generated by the microscope is limited by the PSF. Resolution in the digital image (on which we make our measurements) may be further limited by the sampling of the optical image with

![Image of pixel sizes and magnifications](image)

**FIGURE 1.6**

Pixel size, SNR, and resolution. Comparison of images of the same phalloidin-stained cell acquired with identical exposure settings, using different magnifications and different CCD cameras. Images collected with a (A) 60×1.4 NA objective lens and (B) a 100×1.4 NA objective lens, using the same interline CCD camera based on the Sony ICX285 chip (6.45 μm×6.45 μm pixels) that has been central to CCD cameras from many different manufacturers for well over a decade. Larger magnification (B) results in spreading of light over a larger area, thus less signal and higher noise. (C) Image acquired with a next-generation CCD camera (Sony ICX674 chip) with smaller (4.54 μm×4.54 μm) pixels illustrating an apparent increase in SNR and image resolution. Images at the top (inset in C) show a zoomed region of interest from each of the full-frame images illustrating sampling of the specimen by the different effective pixel sizes. Text refers to the following (from top to bottom): CCD chip, size of CCD array in pixels, objective magnification, and effective pixel size.
the detector (Stelzer, 1998). The resolution of a digital image acquired with a CCD or sCMOS camera depends on the physical size of the photodiodes that make up the chip, while the resolution of digital images created with point scanning confocal microscopes is determined by the area of the specimen that is scanned per pixel. In either case, if the pixel size is too large relative to the size of the object of interest, the optical image will be undersampled and detail will be lost (Fig. 1.5E and F). When using detectors with detector elements of fixed size, magnification in the microscope or in front of the detector can be used to adjust the pixel size. However, there is a trade-off between resolution of the digital image and signal intensity, since increasing magnification alone decreases image intensity as smaller pixels generally collect fewer photons (Fig. 1.6). In live cell imaging, depending on the experimental question, it can be favorable to sacrifice resolution to increase image SNR and/or decrease phototoxicity (Chapter 5).

How does the optical resolution limit affect our ability to quantify in fluorescence microscopy? The size of an object that is below the resolution limit cannot be accurately measured with the light microscope. However, objects that are below the resolution limit can be detected and an image of the object formed by the microscope if the imaging system is sensitive enough and the object is bright enough (Inoué, 1989). While the size of the object in the image will be inaccurate, the centroid of a high SNR image of the object can be used to locate the object with nanometer precision, far beyond the resolution limit (Inoué, 1989; Yildiz & Selvin, 2009). This concept is the basis for superresolution localization microscopy methods such as PALM and STORM (Chapters 14 and 15; Dempsey, 2013). In fluorescence microscopy, the resolution limit does not limit our ability to accurately count fluorescently labeled objects, even if the objects are below the resolution limit (Joglekar et al., 2008; Wu, 2005). If the objects are all of similar size and the intensity of one object can be accurately determined, then intensity values can be used to count multiple objects that are too close to one another to spatially resolve (Chapter 19).

1.5.2 3D SAMPLING

When making measurements of diffraction-limited objects (objects whose size is at or below the resolution limit of the microscope), small changes in focus will have a dramatic effect on the intensity of the object (Hiraoka et al., 1990; Stelzer, 1998). The image of a diffraction-limited object will have a Gaussian distribution of intensity along the optical axis of the microscope due to the PSF, with the width of the Gaussian decreasing with objective NA and wavelength of light (Chapter 10). When using a high NA objective lens to image a diffraction-limited object attached to the coverslip surface, focusing only 100 nm away from the peak of the Gaussian will result in \( \sim 10 \times \) decrease in maximum intensity of the object, clearly an unacceptable level of error (Joglekar et al., 2008; Stelzer, 1998). The ideal approach to accurately measuring the intensity of diffraction-limited objects is to collect a 3D \( z \)-series of images with a very small step size (e.g., 50 nm) using a high NA objective lens (Chapter 2). Specimen dynamics, photobleaching, and phototoxicity often make this approach impossible for live cell work. However, if a sufficient number of diffraction-limited
objects are sampled with a larger step size and averaged together, the error in intensity measurements can be reduced to an acceptable level (Joglekar et al., 2008).

1.5.3 TEMPORAL SAMPLING

To accurately measure changes in intensity or localization of objects over time, one must consider temporal sampling. There are two issues to consider with regard to temporal sampling. The first is how frequently an image is collected during the course of a time-lapse acquisition. The same principles as in spatial sampling apply. If dynamics are temporally undersampled, changing the rate of acquisition will change the results of your analysis quite dramatically (Fig. 1.5). A good example is the acquisition rate dependency of microtubule polymerization dynamics measurements (Gierke, Kumar, & Wittmann, 2010). An ideal solution to this problem would be to decrease the time between acquisitions until a point is reached at which analysis results no longer change. Unfortunately, as in spatial sampling, this is often not possible. Temporal oversampling (i.e., taking images too frequently) is also problematic as it can become very difficult to detect rare events because the specimen may photo-bleach before an event occurs.

The second issue with regard to temporal sampling is how long it takes to collect images at each time point. Each image is essentially a temporal integral of specimen dynamics over the exposure time. Objects that move in one direction during the course of the exposure time, for example, will appear elongated on the image; for example, spherical vesicles can appear tubular. In addition, the fluorescence intensity of the elongated object will be lower, since it will be spread out over more pixels than if the object had remained stationary. Thus, especially for rapid processes, it is beneficial to decrease exposure time as much as possible, which may require increased excitation light intensity (Chapter 5). Temporal averaging is particularly important to think about when collecting z-series of moving objects over time.

1.6 POSTACQUISITION CORRECTIONS

1.6.1 BACKGROUND SUBTRACTION

The pixel intensity values in a digital image of a fluorescent specimen are a sum of both the signal and the background coming from that region of the specimen (Fig. 1.2B). In addition, detectors apply a constant offset value to each pixel in the image to avoid signal clipping due to image noise (Chapter 3). To make an accurate measurement of the signal of interest, background must be subtracted. The best approach to determining the background depends on the specimen, but in most cases, a local background measurement will give the most accurate results. In a local background subtraction, the background is measured in a region directly adjacent to or surrounding each region of interest (ROI; see Chapter 18 for an example), as opposed to making one measurement of background and subtracting this value from all ROIs. Local background measurements minimize error due to
inhomogeneity in the intracellular background levels (for subcellular measurements) or in the medium surrounding the cells (for whole cell measurements). Background measurements should be made on a large enough number of pixels to average out variation and noise; try various size regions to see how the average value changes. Precise background subtraction is especially crucial in ratiometric imaging (e.g., FRET or ratiometric probes measurements of Ca$^{2+}$ or pH; Chapter 24) because

$$\frac{I_{l_1}}{I_{l_2}} = \frac{(I_{l_1} - I_{\text{bkg}})}{(I_{l_2} - I_{\text{bkg}})}$$

Background subtraction should not be done by image arithmetic because intensity variations due to noise will result in pixels with negative values, which cannot be represented in most image formats and will result in signal clipping (i.e., the value of negative pixels will become zero). This results in distortion of intensity measurements. It is best to separately determine the intensities of ROIs and background regions and export these values to a spreadsheet program for calculations.

### 1.6.2 FLAT-FIELD CORRECTION

Fluorescence intensity is proportional to the intensity of illumination (until illumination intensity is high enough that fluorophore ground-state depletion is reached). Therefore, uneven illumination across the field of view of the microscope will result in uneven fluorescence and thus in irreproducible intensity measurements at different positions within the image. The intensity of illumination across the field of view can be measured using established protocols (Model, 2006). If the level of unevenness of illumination is unacceptable, then effort should be made to align the illumination light source (Salmon & Canman, 2001). Despite best efforts at alignment, however, the evenness of illumination in most microscopes will be less than ideal (Chapter 9) and sometimes to an extent that may introduce significant error in measurements of fluorescence intensity.

Flat-field correction (aka shading correction) can be used to correct for uneven illumination across the field of view but requires a good reference image, $I_{\text{ref}}$, of an evenly fluorescent specimen. Saturated fluorescent dye solutions can be used for this purpose (Model, 2006; Model & Blank, 2008), but great care must be taken to ensure that the reference image represents the illumination pattern and not inhomogeneity in the dye solution, or imperfect alignment of the test slide perpendicular to the optical axis. Averaging many different fields of view together can be helpful in creating an accurate reference image.

Flat-field correction normalizes all pixels in an image to a reference value by division of each pixel intensity value of the acquired image, $I_{\text{image}}$, by the corresponding pixel intensity value of the reference image, $I_{\text{ref}}$. Both pixel intensities need to be background-corrected, where $I_{\text{bkg}}$ is a dark image collected without any light sent to the camera (see Chapter 3). Finally, the resulting pixel intensity is multiplied with a
scaling factor (e.g., the average background-corrected intensity of $I_{\text{ref}}$) in order to obtain a similar range of intensity values as in the original image:

$$I(x,y) = \frac{I_{\text{image}}(x,y) - I_{\text{bkg}}(x,y)}{I_{\text{ref}}(x,y) - I_{\text{bkg}}(x,y)} \times \sum_{x,y} \frac{(I_{\text{ref}}(x,y) - I_{\text{bkg}}(x,y))}{xy}$$

The entire calculation needs to be done in floating point math for each pixel before outputting the corrected image, to avoid data distortion due to negative pixel values in the background subtraction steps as well as rounding errors. Thus, this is quite complicated to do right on a routine basis. However, flat-field correction is not always necessary. In practice, other sources of error frequently influence intensity measurements in biological specimens more severely than uneven illumination. Thus, it may be acceptable to restrict intensity measurements to similar areas near the center of the detector field of view where illumination variations are small, measure many specimens, and accept the resulting variation as part of the measurement error.

### 1.6.3 Photobleaching

Photobleaching is the irreversible destruction of a fluorophore, which occurs when the fluorophore is in the excited state. When making measurements over time, every effort should be made to minimize photobleaching over the course of the acquisition, but this is likely not completely possible. Photobleaching can be measured and if necessary corrected for by normalizing measured intensities to the intensity of a known structure. In the simplest case, this can be the intensity of the entire cell, $I_{\text{cell}}$:

$$I(t) = I_{\text{image}}(t) \times \frac{I_{\text{cell}}(0)}{I_{\text{cell}}(t)}$$

However, this assumes that there is no significant change of the total cell fluorescence due to other reasons (e.g., focus drift or the cell moving in or out of the field of view).

### 1.6.4 Storing and Processing Images for Quantitation

Images to be used for quantitation should be stored in the original file format generated by the image acquisition software (Cox, 2006). This file format will contain all of the metadata on acquisition parameters that can be very useful to have on hand during processing and analysis. Metadata may not match the acquisition settings you entered into the software. For example, you may find that the actual time between images acquired during a time-lapse experiment or the spacing between planes in a z-series is different than the interval you set, due to speed or variability of the hardware. However, it is important not to blindly trust metadata. Imaging software does not a priori know most hardware settings, so they must be set up and maintained correctly. For example, pixel sizes measured for a particular objective lens will only
be correct if that objective lens was screwed into the position in the objective turret associated with the pixel size calibration file for this objective.

If you find it necessary to convert from a proprietary file type to a standard file type, be sure to choose a standard file type that preserves the bit depth of the original file and does not use lossy compression (Cox, 2006). Tagged image file format (TIFF) is a standard format that can be read by any imaging software. Avoid file types that change intensity values in the image (e.g., JPEG), thereby rendering your images useless for quantitation. Be careful with pseudocoloring as well; depending on how the pseudocoloring is applied, it may change the intensity values in an image. For example, when creating a 24-bit color image from three 12-bit monochrome images, intensity values are changed as each monochrome image is converted from 12 bit to 8 bits. When image processing, filtering, etc., are used to aid in image segmentation (identification of the pixels in the image that will be measured), intensity measurements should still be made on the original or corrected (e.g., flat-field) files.

1.7 MAKING COMPROMISES

While testing acquisition parameters, you may find it impossible to do the experiment you imagined. In nearly every quantitative fluorescence microscopy experiment, compromises must be made. The key is to make deliberate and thoughtful compromises that minimize impact on the experimental results.

Let us say you want to measure changes in mitochondria content in cells. One approach would be to collect high-resolution 3D z-series images of cells, to segment individual mitochondria in each focal plane, and to measure the size/shape/intensity of each. This approach values spatial resolution over temporal resolution and minimizing photodamage. Another approach would be to collect single 2D lower-resolution images and to measure the area/intensity of the mitochondria in mass. This approach sacrifices measurements on individual mitochondria to gain higher temporal resolution and lessen photodamage.

But what if you want it all—high spatial and temporal resolution—in a specimen that will not allow it? This can sometimes be achieved by performing different sets of experiments using different sets of compromises and then combining the results after analysis.

Never compromise when it comes to health of the specimen. Biologically relevant measurements simply cannot be made on cells that are highly stressed or dying, and fluorescence illumination in combination with biproducts of the fluorescence reaction can easily lead to both (Artifacts of light, 2013). Monitor the health of the specimen using transmitted light microscopy (e.g., phase or DIC), either by collecting a transmitted light image at each time point (if time allows) or by carefully inspecting the cells before and after imaging. Transmitted light microscopy is preferable since the fluorescence channel is reporting only the labeled protein. However, the localization of a single protein may appear to be “normal” even while a cell is blebbing, retracting, and dying.
1.8 **COMMUNICATING YOUR RESULTS**

*Every* quantitative measurement has a level of uncertainty that must be reported ([Krzywinski & Altman, 2013a](#)). Never present a histogram or graph without also including appropriate statistics, and also be sure to report what type of statistics you are showing ([Dukes & Sullivan, 2008; Krzywinski & Altman, 2013c, 2014a](#)). Best practice is to include as much information as possible in the reported data and show either a scatter plot of the entire data set, or if this is impractical, show box-and-whisker plots that allow the reader to evaluate the underlying data distribution ([Krzywinski & Altman, 2014b](#)). The common bar graph with standard error of the mean error bars achieves the opposite and highlights small and possibly unimportant differences of the mean while obfuscating the true nature of the underlying data ([Cumming, Fidler, & Vaux, 2007](#)). If inferential error bars are used, confidence intervals are the most informative ([Krzywinski & Altman, 2013b](#)). It is also important to note that more frequently than not, data from quantitative imaging experiments are not normally distributed ([Krzywinski & Altman, 2013a](#)). It can thus be quite meaningless to show means and standard deviations. As quantitation has become increasingly more important for biological research, several journals have responded by providing excellent reviews on error and basic statistics. The Nature Methods “Points of Significance” series is a particularly useful set of reviews (referenced throughout this paragraph).

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**REFERENCES**


