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TECHNIQUES FOR MOLECULAR ANALYSIS

Imaging the live plant cell

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Summary

Observing a biological event as it unfolds in the living cell provides unique insight into the nature of the phenomenon under study. Capturing live cell data differs from imaging fixed preparations because living plants respond to the intense light used in the imaging process. In addition, live plant cells are inherently thick specimens containing colored and fluorescent molecules often removed when the plant is fixed and sectioned. For fixed cells, the straightforward goal is to maximize contrast and resolution. For live cell imaging, maximizing contrast and resolution will probably damage the specimen or rapidly bleach the probe. Therefore, the goals are different. Live cell imaging seeks a balance between image quality and the information content that comes with increasing contrast and resolution. That 'lousy' live cell image may contain all the information needed to answer the question being posed –provided the investigator properly framed the question and imaged the cells appropriately. Successful data collection from live cells requires developing a specimen-mounting protocol, careful selection and alignment of microscope components, and a clear understanding of how the microscope system generates contrast and resolution. This paper discusses general aspects of modern live cell imaging and the special considerations for imaging live plant specimens.

Keywords: live cell imaging, microscopy, digital imaging, confocal microscopy.

Introduction

Video microscopy changed our view of the cell dramatically (Allen and Allen, 1981; Allen *et al.*, 1981; Inoue, 1981). Watching still images of mitosis, cell division and other cytoplasmic arrangements come to life as a movie revealed the importance of position and timing in understanding cellular mechanisms. Genetically encoded optical probes (Chalfie *et al.*, 1994) have similarly changed our scientific viewpoint by moving our notions of protein localization beyond those formed from static immunofluorescence images (Weber and Osborn, 1985). Visualizing the *in vivo* movement of proteins has provided temporal and spatial context for protein function, often illuminating critical aspects of the mechanism under study.

Imaging live cells brings with it new opportunities and technical challenges. The inner workings of the plant cell stream, diffuse and deform with apparent enthusiasm. And unlike cultured animal cells, the thickness of a typical plant cell exceeds the focal depth of a high magnification lens by 20–100 times. Therefore, capturing information about protein or organelle localizations throughout the cell requires trade-offs between data acquisition speed, contrast and resolution. Commercial imaging devices now collect enough data, fast enough, for limited 3D –time-lapse experiments, although the software for quantitative evaluation lags far behind (Thomann *et al.*, 2003).

Observing where proteins localize is great, but in the live cell, we also want to know *when* the protein is active. Fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM) techniques give us a chance to ask questions about *in vivo* binding events and protein activity, moving beyond the 'guilt by association' arguments relating localization to function. The practical application of FRET and other fluorescence technologies must involve an understanding that the cell wall and plastids fluoresce over a broad and seemingly unpredictable range of colors, and contain crystalline elements that interact with light.

Finally, proteins are only part of story. Visualizing what is not directly encoded by the genome (e.g. lipids, metabolites,

extracellular matrix polymers) is becoming increasingly important for understanding cellular mechanisms. All of these technologies are predicated on the investigator solving the problem of how to image their specimen in the living state.

The goal of this paper is to provide information about collecting image data from living plant cells. Despite the best efforts of most manufacturers, the microscope is not yet available in kit form with simple instructions about how to get live cell data. Most investigators have a compound microscope or access to a more elaborate system in a departmental facility. Acquiring live cell data from that device requires evaluating the equipment and creating a strategy for preserving the biological process while capturing the needed information. The first section of this paper outlines several methods for mounting and culturing plant specimens on the microscope stage. The following three sections develop general background information about how contrast and resolution relate to the elements of the imaging system. Section 5 surveys microscopy techniques for quantifying molecular distributions or activities within cells. The final section describes how to set up and evaluate the microscope for live cell data collection.

Before you start

Most imaging projects begin with the goal of protein localization. The common post-genomic plan is to make the GFP fusion(s), transform plants, and ask, 'Is the protein where it is *supposed* to be?' After some squinting and looking around, a decision is made about the localization and images are taken to support that decision. Later, looking over the data, questions arise, such as 'Why is it also there?', 'How much does the mutant really differ from wild-type?', 'Hmm. Expression artifact?', 'Is this development/cell cycle/time of day dependent?', or a personal favorite 'What organelle is that?'. The weight of our collective observations shows that most proteins lead a complex lifestyle. They localize to multiple places, often perform multiple tasks, and develop bad habits when improperly expressed. Thus, even accomplishing the apparently modest goal of determining whether the protein localizes to an hypothesized location is seldom straightforward.

Relating a carefully made observation from the microscope has a well-deserved place in science. It is, however, no substitute for an experimental test of hypotheses. Images, especially really compelling ones that reinforce your ideas, too often guide our thinking about biological mechanisms. Science requires a systematic test of a hypothesis, showing a pattern of observations that either support or refute the hypothesis.

With that in mind, the first stage in planning any experiment is to clearly state the hypothesis. 'Where is my protein?' is not actually a hypothesis. Proposing that the protein localizes to the nuclear envelope for example is a better start because the proposal can be tested quantitatively against the established properties of other proteins (Cyr *et al.*, 2006). My point is that the burden of proof for image-based data lies not simply in stating that 'it' was observed x times out of n trials. Rather, the burden lies in demonstrating with what accuracy 'it' is identified, and with what precision you can discriminate between control and experimental conditions.

1. Developing culture conditions for live cell imaging

The first task in live cell imaging is culturing the specimen on the microscope stage. Plants sense light fluence, frequency and polarity, temperature changes, touch, osmolarity, humidity, electric fields and gravity, amongst other variables (see Buchanan *et al.*, 2000). Plants also have strong circadian cycles of physiological behavior and gene expression. Most environmental stimuli have short-term consequences and longer-term effects as the plant regains equilibrium with its environment. For example, even small osmotic changes can stimulate an efflux of peroxide radicals (Park *et al.*, 2000), influencing growth behavior, increasing autofluorescence, and inducing later gene expression. The practical solution to reducing experimental variation is to establish a defined protocol for your experimental system, where specimen recovery time has been characterized and controls have been run to demonstrate that the biology of interest is not compromised by the culturing conditions or the imaging process. Designing a protocol will include media selection, creating an imaging chamber, and specimen mounting.

Selecting and characterizing a culture medium

Imaging subcellular structures nearly always requires that the specimen be submerged in liquid medium. Lenses designed for high-resolution imaging rely on the optical properties of the liquid to conduct light into the microscope. The simple rule for wet mounting specimens is to use the same medium for growth and imaging whenever possible. Defined plant growth and culture media [e.g. Murashige and Skoog (MS), Schenk and Hildebrandt (HS), Gamborg's B-5, etc.] typically have a good osmotic balance but may be unsuitable for specific imaging experiments dependent upon supplier, batch and amendments. The major considerations are the presence of colored or fluorescent components, light-sensitive chemicals, and additions that alter the refractive index. If liquid media cannot be used, be keenly aware that the imaging process will heat and dehydrate the specimen.

Scan the liquid medium in both a spectrofluorometer and a scanning spectrometer from 400 to 650 nm wavelengths. The spectra should be absent of peaks and not

significantly high in background when compared to tap water. If peaks appear within the excitation or emission range, this can degrade image quality. Peaks appear due to added hormones, vitamins, fertilizers and buffer impurities amongst other factors. If it is biologically reasonable to remove the component from the medium, eliminate it.

Many plant hormones, media additions and small molecule drugs convert to another compound when exposed to strong irradiance, especially light below 400 nm wavelength. Photo-conversion of media components can lead to unexpected, time-dependent effects.

Sugars, or other higher molecular weight compounds, at >5% w/v, may alter the refractive index. Keep this in mind when choosing an objective lens and immersion medium (see below). When complex media cannot be used, plain MS medium (Murashige and Skoog, 1962), 0.5× MS or artificial pond water (APW: 0.1 M NaCl, 0.1 M KCl and 0.1 M $CaCl_2$) are good starts.

Low melting point agarose and other gelling agents for embedding tissue culture cells (Damm *et al.*, 1989) are somewhat destructive to contrast-generating techniques, such as differential interference contrast (DIC), and often contain light-absorbing or fluorescent compounds that interfere with fluorescence imaging. Scanning a dilute solution of the medium, as noted above, is recommended before use. If the gelling agent differs from that used in culture, it is important to demonstrate that the agent does not cause a biological response that will influence your experiments.

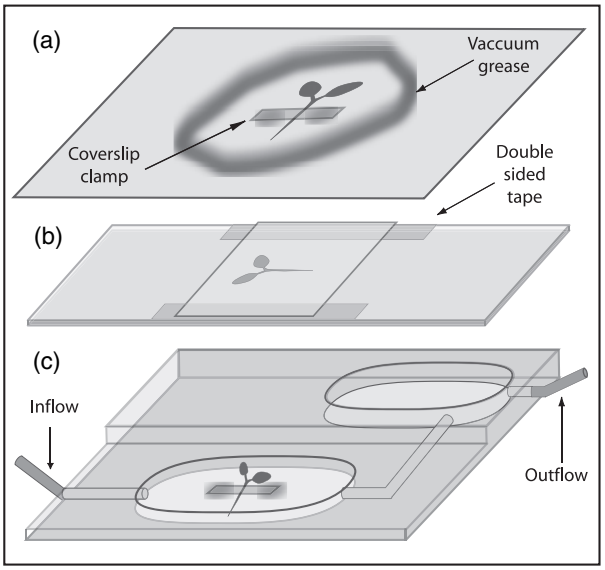

Figure 1. Imaging chambers and mounts.
(a) A large format glass coverslip with a ring of vacuum grease used as an imaging chamber. Clamping the specimen with a rectangular piece of coverglass and vacuum grease prevents specimen drift in the open chamber.
(b) Strips of double-sided adhesive tape, applied in two layers to either side of a glass slide, create a flow chamber with a glass coverslip.
(c) Perfusion chambers made from machined plastic have a specimen chamber and a separate chamber for media outflow. Seedlings are shown for illustration, but leaf tissue, root explants and tissue culture cells can be mounted in a similar manner.

Specimen mounting and perfusion

The complexity of the specimen mount depends upon specimen type and experimental needs (Figure 1). For short-term experiments (<30 min), a 60 × 50 mm glass number 1.5 thickness coverslip, with a ring of vacuum grease forming a bath, serves as an excellent 'culture system'. The vacuum grease should form a wall at least 2 mm high and trace an outline large enough to accommodate the specimen. A minimum of 0.5 ml liquid should be used to avoid rapid changes in buffer concentration due to evaporation. Drying down 500 μl of 10 μg ml^{-1} poly-L-lysine on a glass surface, prior to specimen transfer into the bath, will adhere tissue culture cells, but does not hold larger specimens. Placing a second coverslip over a larger specimen will hold it in place, provided a good seal is made with the ring of grease forming the bath. A second method for holding specimens, useful when an open bath is necessary, is to use a rectangular strip of coverslip (5 × 22 mm cut with a diamond scribe) attached with vacuum grease, to span the plant axis and act as a specimen clamp (Figure 1a). For small plants (e.g. Arabidopsis seedlings), tissue culture cells and excised embryos, a chamber can be formed using double-sided adhesive tape (approximately 60 μm per layer) as a spacer between coverslips, or a coverslip and a glass slide (Figure 1b). Medium can be drawn through this chamber by addition at one side and wicking with a tissue at the other. Obviously, crushing the plant in any of these chambers is a stress inducer.

Longer duration experiments benefit greatly from medium replacement. Media exchange prevents the local elevation of gases, hormones, ions and secondary metabolites that influence specimen stress levels. Manual exchange with a pipette physically disturbs the specimen and can be stressful if significant evaporation occurs between additions. Constant perfusion can be accomplished by gravity flow, peristaltic pump, or most effectively, through use of a syringe pump. Coupling two syringe pumps to the chamber through a simple manifold provides constant, reproducible flow with the option of creating gradients or introducing media amendments in a controlled manner. Liquid outflow depends upon chamber design, but is usually accomplished by gravity flow, vacuum line or a peristaltic pump. The experimental protocol and chamber volume will dictate the minimum flow rate, while the maximum flow rate should not physically disturb the specimen. Introducing a known volume and concentration of dye at the chamber inlet and imaging the change in dye intensity as it passes through the

system is informative for setting a minimum flow rate. The rate needs to be fast enough to prevent dye from accumulating in pockets around the specimen that persist when the remainder of the chamber is devoid of dye.

Perfusion can be set up using the large-format coverslip. Flexible tubing of 2–3 mm internal diameter leading into an open-topped bath delivers the medium, while tubing of the same dimension evacuates the medium from the opposite side. The flow rates are typically inconsistent between experiments due to differences in bath volume and the difficulty in matching outflow and inflow rates. When using an upright microscope stand (without a 'dipping' lens, explained below), or when performing high-resolution transmitted light imaging on an inverted stand, the specimen must be mounted between glass surfaces for using the objective and condenser lenses. Setting up perfusion under these circumstances using a coverglass chamber can be performed by creating an elliptical bath, where a coverslip over the middle of the bath covers the specimen and the perfusion lines access the medium on either side.

Most commercial, pre-formed chambers are designed for mammalian tissue culture cells or tissue slices. Chambers developed for recording electrophysiological measurements are often large enough for use with plant seedlings, tissue peels and tissue culture cells. The key design features are an inlet tube, a defined chamber volume, and a second chamber that serves as a reservoir for the outflow (Figure 1c). Typically, the chamber is affixed to a large format coverslip by vacuum grease, and the entire chamber can be taped or otherwise affixed to the microscope stage. Liquid enters through the inflow tube, flowing over the specimen in a laminar fashion. Liquid either spills over a barrier into the second chamber or the outflow tube is held slightly above the coverslip level. In either case, the second chamber makes it far less likely that the specimen chamber will ever go dry, spoiling the experiment. These chambers come in both open and closed versions, where a coverslip placed over the specimen chamber in the closed version facilitates use of an upright microscope and transmitted light imaging for inverted stands. Creating a perfusion system for larger plant specimens will require construction of your own chamber apparatus.

Temperature control

Controlling temperature on the microscope stage is difficult. Rapid or cycling temperature shifts in the microscope room, often found in air-conditioned or heated rooms where the thermostat is elsewhere, can be a significant problem for maintaining focus. They may also interfere with the biology of the plant system. As a general rule, keeping the room and microscope at a constant temperature is a better plan than attempting to keep the specimen at a defined temperature in a room that is fluctuating. Fitting part of the microscope, including the stage, with an air-conditioned plexiglass enclosure works reasonably well to buffer the specimen against rapid temperature changes. Temperature-controlled specimen chambers can be purchased that provide thermostat-controlled updating of the medium. Using these stages is only recommended if temperature shifts are desired for experimental purposes. Perfusion with medium from a temperature-controlled source is an effective method for regulating specimen temperature or to introduce rapid temperature shifts, provided the flow rate and total volume are high enough to accommodate the heat or cold transfer under your imaging conditions.

Creating a mounting protocol

Sterilize the chamber with ethanol or bleach, if required. Clean a large format number1.5 coverslip with lint-free lens tissue on both sides. For sterile preparations, apply ethanol to the coverglass and gently flame to get rid of residual dust, bacteria, oils or other debris. Do not continually heat the coverslip or it will deform. Apply vacuum grease to the coverglass, using a 5-ml syringe to create a bath or for adhering the coverslip to a chamber. If desired, cut a section of 22 mm coverslip for use as a specimen clamp and store in ethanol for later use. For adhering tissue culture cells or embryos to a slide using poly-L-lysine, treat coverslips for 5 min with 0.1 N HCl, in lieu of alcohol, and rinse with distilled water prior to application of poly-L-lysine.

Move the specimen from the growth culture to the chamber in a manner that avoids rapid changes in temperature or humidity. For specimens grown on agar plates, wetting the specimen with medium prior to transfer, especially if working in a laminar flow hood, will help prevent dehydration. For leaf tissue or tissue peels, submerge the tissue in medium for several minutes to relieve it of trapped gas bubbles. For clamping, apply two spots of grease on to the coverglass, spanning where the plant will reside. Pipette a small amount of medium on the coverglass just prior to transfer of the plant. Carefully place the specimen(s) on the coverglass, and, if required, attach the second coverslip or coverslip clamp with forceps. Add medium to bring the mounting chamber up to volume.

Equilibrate the plant for a minimum of 15 min prior to starting any experimental measurements, especially if using perfusion. Take note of any property of the specimen that could have an effect on the biology you wish to examine. Record the details and timing of the specimen mount either in a database or on a dedicated worksheet with large enough print for easy viewing in the dim microscope room. Where practical, replicate the conditions and timing of your protocol explicitly. The details often matter later, even if they seem initially trivial.

2. Capturing information in a digital image

The one inescapable rule for imaging live cells is that you must limit the radiation (light) used to illuminate the specimen. Unlike fixed material, the massive photon flux used for fluorescence and brightfield imaging will affect the biology of the specimen, especially a plant cell attuned to light collection. Evolution has not developed an effective protection against light flux that is 100 to 10 000 times greater than normal terrestrial levels. Plants contain an extraordinary number of molecules that absorb, redirect, or serve to interpret incident light. Activating or damaging these molecules can affect physiological processes as well as initiate stress responses in the plant, feeding back directly on the biology under study. Beyond the intrinsic biological issues, high light exposure photobleaches the dyes and fluorescent proteins used for contrast generation. Photobleaching ruins the signal and introduces free radicals into the cell (Bensasson *et al.*, 1993; Dixit and Cyr, 2003). Hence, the amount of illumination used to create an image must be balanced against specimen damage and signal loss over the duration of the experiment. Accomplishing that goal requires an understanding of what constitutes an image and how the elements of different microscope systems contribute to image formation.

The good image

We begin by defining an image as a distribution of intensities and a feature as a subset of image intensities that forms a pattern. A feature may be simple, like a spot, or complex, like the collection of spots that make up a recognizable face. Our confidence in the feature's identity is based upon how well the pattern in the image matches our criteria for an object pattern, and the uniqueness of the pattern within the image. Finding a spot, for example, requires detecting an intensity difference against a background. Finding a specific spot, in a field of spots, calls for more information about relative intensity or position. Finally, identifying a feature with the complexity of a face requires finding subtle intensity changes, grouped into a spatially related context. The important point is that features of different complexity require different amounts of information for identification. The goal in imaging a fixed specimen is to maximize the information content in the image. In contrast, a good live cell image contains just enough information to confidently identify the feature of interest. Collecting more information can unnecessarily damage the specimen or probe.

The imaging system uses light to convert a molecular distribution (the specimen) into a representative intensity distribution (the image). In that sense, any measurable difference in image intensity, informs us about the specimen. When probing the specimen with light, the physics of light capture by lenses and the damaging effects of irradiation fundamentally limit the quantity of light used for image formation and the spatial dimensions over which intensity differences can be discriminated. In other words, the optical system puts an upper bound on the amount of information that can be collected from the specimen (Figure 2a). The image detector further limits information collection based on the amount of space and intensity that are grouped together to create each pixel value (Figure 2b). For example, a 10×10 array of pixels obviously has limited information capacity. A 1000×1000 pixel array of the same physical size captures much more spatial information, but each array element collects only 1/10 000th as much light, a massive difference in light sensitivity. Creating the best representation of the specimen, while not damaging the specimen in the process, therefore requires knowledge of the optical limits of the microscope and the compromises available when using electronic image detectors.

Our confidence in the attribution of feature identity relies upon the principles of measurement. As an abstracted example, let's say 50 people measure the same pencil using plastic rulers. The pooled measurement values form a distribution, where the mean value represents the best approximation of pencil length and the spread of the distribution helps us understand how much confidence to have in that averaged value. We are more likely to believe an average from 50 tightly grouped values than an average from five measurements that are broadly spread. The spread of the data, representing the precision of the measurements, is important because a broad, highly asymmetric or multimodal distribution would lower our confidence or suggest a measurement error. The estimate accuracy, how close it comes to the true length value, depends upon how many measurements we take and measurement biases, such as differences in the plastic rulers, participant's attention span, or if some people used the pencil to record their measurements.

Each image in an imaging experiment constitutes a measurement, where the information in the image contributes to the confidence level in your final stated result. Live cell images are often grainy, fuzzy, ill-composed shots that represent the maximum amount of information extractable from the specimen under experimental conditions. Synonymous with the example above, confidence in your claim of feature identity relies on showing many examples of the feature, evaluating the range of feature variance, and demonstrating that the feature does not differ from a positive control. In summary, even if the image is lacking in aesthetic appeal, it is a good image if it contains the information required to detect a feature and further your test of hypotheses.

Contrast and resolution

Live cell imaging involves finding a balance between image quality and limiting the damage to specimen or probe.

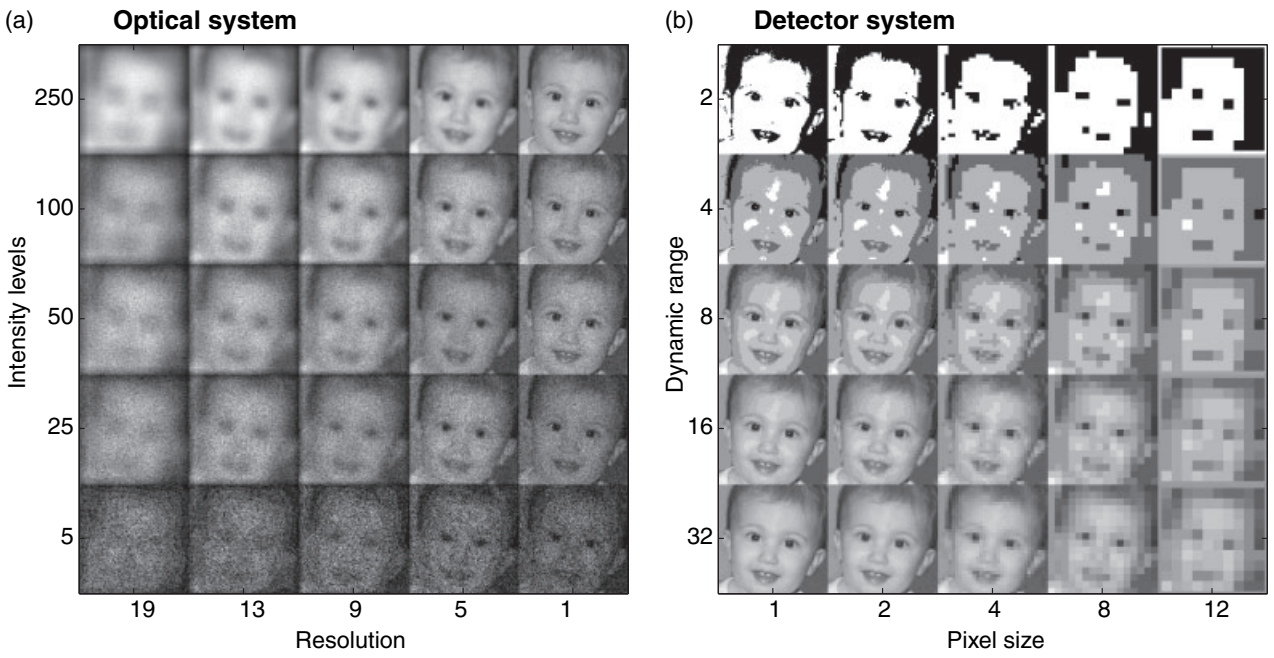

Figure 2. Collecting specimen information is limited first by the optical system and then by the detector.
A human face is used for illustration to more easily relate information content to image quality. The optical system determines the quantity of light available for contrast generation and the physical resolution limit (a). As light intensity increases (a, bottom to top), the photon counting noise becomes less apparent. As optical resolution increases (a, left to right), the general features of a face appear followed by more specific features including a nose, mouth and locks of hair. With more information, we gain confidence in estimating age, sex, mood, number of teeth, and any unique features of the individual. Note how the intensity and resolution work together. Resolution was decremented using Gaussian kernels of the size marked on the vertical axis and noise was added as a square root function of scaled intensity. The detector system takes the information captured by the optical system [upper right in (a) to bottom left of (b)], and samples the data into discrete light levels (b, bottom to top) and spatial elements (b, left to right). As the pixel size increases (b, left to right), feature details are lost until we are no longer certain it is a face. Similarly, as the number of gray levels used to capture the optical signal decreases (b, bottom to top), shading is lost and only highly contrasted features remain. Again, note that the two factors work together in providing information. For live cell imaging, the amount of information collected should be proportional to the amount required for verifying your hypothesis (e.g. this is a face, this is an old face, this is a happy face).

Making a good compromise requires a practical understanding of image quality. To begin, we need to relate the representative units used for describing image quality (pixels and gray levels) to the physical units of specimen measurement (nanometers and photon flux). Ideally, every pixel value in the image would represent the exact photon count coming from a precisely defined area of the specimen, independent from all its neighboring pixels. That is simply not the case. Gray level values and the stated degree to which the picture elements (pixels) define an object's morphology are relative estimators. This relationship is most commonly explained using the concepts of contrast and resolution.

Estimating light intensity

Opening the camera shutter allows light energy to collect in the pixel elements of the electronic detector. If we kept track of the accumulated photon to electron conversions (photoelectrons) over time, it would reveal a Poisson distribution of events. In other words, photon emission has the fundamental property of being stochastic with respect to time. Independent of the photoelectrons (signal), spurious electrons also accumulate randomly in the detector (noise). The stochastic nature of both these events has a defining influence on our ability to put an exact value on light intensity (Figure 3a). If an image is taken with no light provided to the detector, the signal-independent electrons collect and form a distribution, characterized by a mean value and a standard deviation. The mean represents a background and the standard deviation describes the uncertainty, or system noise, imparted to the signal value. When light is applied, photoelectrons and signal-independent electrons are collected but cannot be distinguished from each other. The background value, calculated from a dark image, is often subtracted from every pixel value in the image, but the uncertainty due to the noise (estimated as the standard deviation of the averaged background) cannot be subtracted away. Each detector element contains signal and noise electrons that are converted to an intensity/pixel/gray level value, where the number of electrons converted to each pixel level can be any integer value, including 1.

Let us be clear that each pixel value is only a statistical estimate of the photon flux associated with an area of the specimen. Since relative intensity levels constitute the data we want, it is important to know how much confidence to

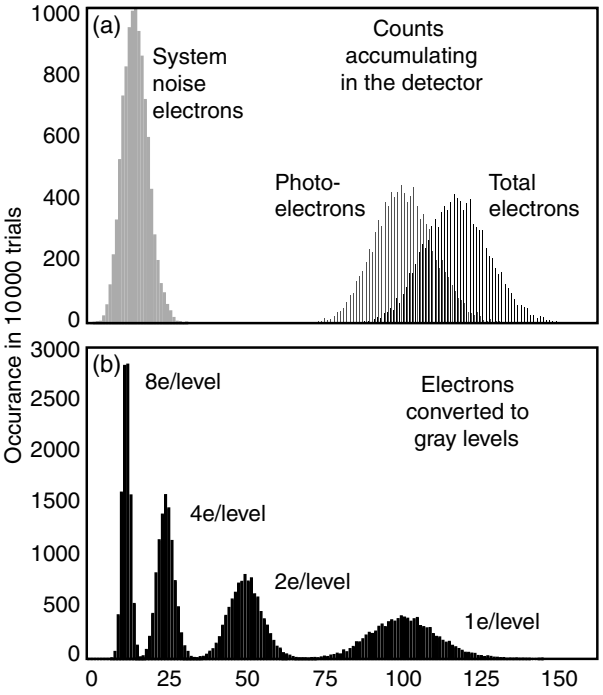

Figure 3. Signal and noise electrons appear randomly with respect to time. (a) A simulated distribution of the counts collected in a single detector element over 10 000 exposures. The signal-independent electrons, representing dark current and read-out noise, form a distribution of system noise with an average of 16 and a standard deviation of 4. The distribution of photoelectrons forms a similar distribution with an average of 100 and standard deviation of 10. The detector element records the combined photoelectrons + noise electrons distribution with an average of 10 and a standard deviation between 10 and 11. (b) Conversion to a gray level using 1, 2, 4 or 8 electrons per gray level. Increasing the number of electrons per gray level lowers sensitivity but increases confidence in the intensity value.

have in the intensity estimates. Since each pixel value is a single measurement of a Poisson process, the odds of that single pixel value representing the long-term average photon flux are grossly approximated by a normal (Gaussian) distribution, with the first standard deviation equal to the square root of the pixel value. That square root relationship is important. It defines the maximum confidence we could have in any single measurement of photon flux, even if we eliminated all of the electronic noise in the system.

The over-arching point is that when we want to quantify the amount of light originating within an area of the specimen, we can only estimate that value, and our confidence in the estimate depends upon the magnitude of the signal and the noise in the measurement system. For that reason, a common means of specifying data quality from a detector is the signal to noise ratio (SNR). A SNR of 2.7:1 has been suggested as a lower limit for detecting signal over noise (Colarusso and Spring, 2003). For example, let us consider two consecutive exposures, one with the shutter closed and the other with the shutter open. Evaluating the dark image, the average pixel value is 50, with a standard deviation of 7. Looking at the specimen image, we find a pixel with a value of 114, well above the background. The signal is the proposed number of photoelectron conversions, calculated as the pixel value minus the background ($114 - 50 = 64$). The total noise equals the system noise, measured above as 7 (and also estimated as the square root of 50), added in quadrature to the photon counting noise, which is equal to the square root of 64. Simply dividing the signal (64) by the noise [$(7^2 + 8^2)^{0.5} = 10.6$] yields an SNR for the pixel value of about 6. When the pixel values fall below 73, where the SNR approaches 2.7, the odds that the value is related to statistical fluctuation, instead of signal, are uncomfortably high. As a rule of thumb, each SNR unit roughly equates to one standard deviation from the mean signal value, and 2.7 standard deviations implies a reasonable confidence for detecting signal.

Intensity values are almost always created from multiple (photo)electrons (Figure 3a), meaning that the distribution of pixel values no longer forms a Poisson distribution with the standard deviation (noise estimate) equal to the square root of the mean. Therefore, a measured standard deviation is a safer estimator for noise. For live cell work, and especially live plant cells, there is nearly always light collected that is unrelated to the feature of interest (autofluorescence, etc.). As more unwanted light is added to the image from autofluorescence, internal reflections or out-of-focus elements, the feature becomes more difficult to identify.

In practice, the unwanted light and electronic noise are estimated together by calculating the average value and standard deviation from an area near the specimen instead of from a frame taken with no light to the camera. That background can change from image to image and should be taken from enough pixels (>36) to create a robust estimate for the mean and standard deviation. The signal is generally calculated as the average of a few closely associated pixels minus the previously calculated background value. The noise approximation is created as above, by adding the noise components in quadrature, using the square root of the signal value for the noise estimate when necessary. The final SNR now takes into consideration the effect of the unwanted light. If stray light increased the background value in the above example from 50 to 500, it would no longer be possible to detect the signal of interest (64) because the noise [$(500 + 64)^{0.5} = 24$] has increased the level of uncertainty for the pixel intensity (SNR <2.7).

Increasing confidence in an intensity estimate is achieved through data pooling or averaging. Obviously, using the averaged value of 50 pixels to represent a claim is a much stronger argument than claiming that a single pixel accurately represents the specimen intensity. Summing or averaging a block of pixels together increases confidence at the expense of spatial resolution. Averaging sequential frames together increases confidence at the expense of temporal resolution. The increase in SNR from spatial or

temporal averaging is generally proportional to the square root of the number of pixels averaged. Therefore, provided the signal is constant and the noise is random, averaging four frames reduces noise by a factor of 2, while averaging 16 pixels reduces the noise by a factor of 4.

Using more than one electron for each gray level increases confidence in the intensity value at the expense of sensitivity. A detector with perfect collection efficiency and ± 4 electrons noise would report 100 photons as 99–101 less than 10% of the time, but taken four electrons at a time, a value of 24–26 is reported about 40% of the time (after background subtraction).

Relating intensity to contrast

Contrast is a relative value and generally refers to black (absence of signal) and white (saturating signal) and the intervening shades of gray. When we view a linear gradient of eight monochromatic intensity steps from black to white, the transitions appear distinct (Figure 4a). As additional shades of gray are added between black and white, our confidence in finding the individual contrast steps falters. We can no longer determine when one shade stops and another starts because the noise generated by our optic and neural processes matches the difference in the intensity step. The number of resolvable steps between black and white defines a contrast range and therefore the number of possible information units available from intensity. The contrast range is determined by the saturation point of the imaging system and the noise associated with each signal intensity.

A typical imaging device has a much larger available contrast range than the light-adapted human eye. Where the

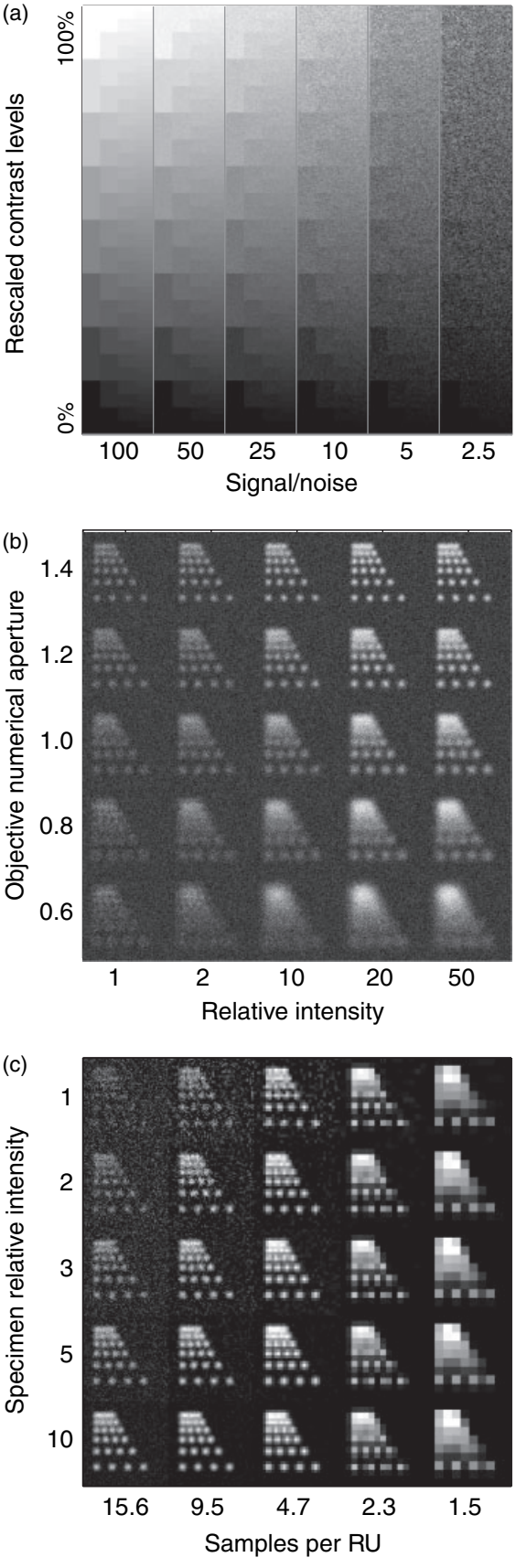

Figure 4. Contrast and spatial resolution.
(a) A linear contrast scale of 8, 16, 32 and 64 steps from no signal (0%) to saturation (100%) has been replicated with a progressive increase in signal-dependent noise. The transitions between steps become increasingly more difficult to identify as the noise level increases. For each panel, signal-dependent noise was added and the intensity was rescaled from the new maximum to zero as if sampled by a noiseless detector.
(b) Spatial resolution is affected by both the lens numerical aperture and the recorded intensity. Spacing of the six points in the first column of each panel represent the resolution limits specified by the five numerical apertures listed on the vertical axis. Points in the first row are spaced just below the resolution limit for a 1.4 NA lens, while points in the remaining rows are at the resolution limit for the five numerical apertures listed. Each image in the first column simulates the use of a different NA lens on the same points. As NA increases, the photons are concentrated into a smaller, brighter spot. As the relative number of photons emitted per point increases, indicated on the horizontal axis, resolving the individual spots becomes easier. Background represents random electron noise.
(c) The relationship between spatial sampling and signal collection. For each panel in the first column, proportionately more simulated photons were collected with the same read-out noise and a 1.4 NA objective lens. The upper left panel represents an SNR of 2. As fewer pixels are used to image the same area, the signal per pixel increases while resolution is lost. When too few pixels are used to sample the resolution unit (RU), points often blur together or form new patterns through a process termed aliasing.

human eye has problems accurately determining more than about 100 shades of gray, scientific-grade CCD cameras can often resolve thousands of shades of gray between saturation and the absence of a signal. Contrast adjustments are often made after the image is taken, by scaling the relative pixel values, using a look-up table (LUT) and software. Scaling the image intensity values to accommodate normal human vision helps us in our visual interpretation of the data, but it does not improve the SNR of the data.

For example, if a monochrome image has 1000 contrast levels and the feature we are looking for is in the brighter portion of the image, it may appear washed out and difficult to identify. This occurs because the 1000 gray levels in the image have been compressed to less than 256 values for presentation on the computer monitor and your eyes can only discern a fraction of that contrast range. If the feature intensity spans 50 contrast steps in the original image between values 900 and 950, it might appear as only 5 or 10 steps on the monitor and look like an igloo in a snowstorm to our eyes. By setting all values in the image below 750 to 0 (black), and rescaling the values between 750 and 1000 to be displayed from 0 to 250 (black to white) on the monitor, the effective contrast will be larger over the contrast range of our feature of interest. We will be better able to identify the feature because the contrast range is better scaled for human perception. If, however, the feature intensity only spans five contrast steps out of 1000 in the original image, scaling the contrast is not going to help in resolving the feature identity.

From the above discussion, we can now put the concept of contrast into perspective with regard to image quality. I have defined contrast as a relative measure of intensity and a contrast range as the number of contrast steps that can be differentiated in the image. Deciding whether two pixels or regions of intensity constitute different contrast or intensity levels boils down to a question of statistical confidence. For example, given the noise properties of the image, could you pick one region as being different from the other more than say 15 times out of 20? This is directly analogous to treating one value as signal and the other as noise, and asking if there is reasonable evidence that the SNR exceeds 2.7. The general goal for live cell imaging is not to get the maximum number of possible contrast steps (the highest 'quality' image), but rather to capture enough contrast to confidently identify the feature of interest without damaging the specimen or probe.

Resolving objects in space

Spatial resolution is defined by the ability to identify an intensity difference between two points (Jonkman *et al.*, 2003). If we imagine two equally bright points moving together in tiny increments, the points are still resolved when the intensity difference in the space between them is greater than that expected for the noise (Figure 4b). That minimum resolvable distance, the resolution limit, is ultimately determined by the physical properties of light and the objective lens. If fluorophores, for example, are concentrated into a progressively smaller area, the resulting spot size stops shrinking in the microscope when it reaches a diameter just larger than the resolution limit of the lens. The spot size is limited by the diffraction properties of the objective lens. The same two points, at the same magnification, appear larger using a low-resolution lens and smaller for a high-resolution lens (Figure 4b). Hence, two points spaced the same distance apart are more likely to be resolved, by the above criteria, with the high-resolution lens. By extension, an edge or a line in a high-resolution system becomes softened in a low-resolution system. As the resolving power increases, spatial variations in contrast become more distinct.

Capturing the available optical resolution in an image is limited by the spatial sampling rate of the detector (Figure 4c). Lets say the physical resolution limit of the optical system is 1 μm and we find two spots placed 3 μm apart. If the spots appear 30 pixels apart in the image, we should see two peaks and a valley, provided we can confidently detect the intensity peaks. If the peaks are imaged only two pixels apart, it becomes nearly impossible to say that the difference in intensity in the intervening pixel is due to a real intensity difference, and not noise. The resolution is under-sampled in the image, and information was lost. If we image two points that are placed 0.25 μm apart, using the same optical system, and magnify the image so that 30 pixels span the area, there is still no way to resolve the points in the image. The over-sampling will not compensate for a lack of resolution in the original image (Stelzer, 1997).

For live cell work, I suggest a sampling rate of 3–8 pixels per resolution unit to collect the majority of the resolved information. The often-suggested Nyquist sampling limit of two pixels per resolution unit is misrepresented for imaging. Nyquist demonstrated that 2.3 discrete samples per minimum frequency component could be used to recapitulate the information in the signal waveform, provided that the correct basis function was employed in the reconstruction (see Young, 1989). Camera-based imaging systems, and most point-scanning systems, use a square grid (Cartesian) pixel array. The signal is integrated between points rather than sampled at discrete points, resulting in bleedthrough or non-independence in sampling for optically resolved points if there are too few pixels. Resolving feature elements in the image that are not parallel or perpendicular to the pixel array requires more sampling, because the pixel centers are proportionately further apart in the Cartesian array. Lastly, pixel intensities are nearly always reproduced as raw values without correction for sampling density or any underlying wavelength or pattern. Magnifying the image to the detector such that only two pixels cover each resolved distance will

not capture all of the available information from the optical system.

Having a resolution limit implies that the distance between two points cannot be measured if it lies below the resolution limit. For example, reporting two features as being 0.25 μm apart, using a lens with a 1 μm resolution limit, is highly suspect. However, a distance between two spots further apart than the resolution limit can be measured with accuracy greater than the resolution limit. The center of a single, luminous point can be estimated to within 10 nm, dependent upon sampling and SNR (Ober *et al.*, 2004). Therefore, the distance between any two luminous points, when more than the resolution limit, can be measured to an accuracy in the tens of nanometers (Inoue, 1989).

Resolution and contrast are interdependent

Contrast and resolution are inextricably linked in the image (Figures 2a and 4b). Resolution is literally defined by the ability to demonstrate contrast between two points. Obviously, generating contrast is required for capturing resolution in the imaging system. Similarly, as optical resolution is changed, the signal intensity per pixel, and thus the uncertainty level in feature identity, will change. The crucial idea is that resolution and contrast must be considered dependently when matching the information content of the image to the requirements for feature identification in the live cell image.

3. Modern imaging systems

Collecting enough light from the specimen and getting light to interfere in an informative manner provides the contrast and resolution needed for feature detection. Biological materials are mostly transparent to light, having few chromatic features or large changes in refractive index for producing contrast. Therefore, many techniques have been invented to enhance the limited contrast available from the specimen or to add contrasting features, such as fluorescence. Before deciding what technique to use for your experiment, there are several general aspects of the imaging system to consider. These include the microscope body style, the objective lens, the illumination system and the image detector.

Microscope body style

Most live cell imaging systems use a fixed stage microscope, more commonly found with inverted microscopes. The fixed stage provides a stable platform for perfusion and micromanipulation equipment, and gross focusing does not disturb the specimen. The inverted microscope has the advantage that open chambers can be used with the specimen flattened against the bottom coverglass. The upright microscope handles immersion lenses better than inverted body styles and has a direct port to the image detector, avoiding minor light losses through side ports. Newer frames often include motorized focusing, filter changing and objective selection. While automation brings convenience, there is nothing inherently better about the optical performance of an automated microscope. Instrumentation for maintaining focus, either laser-based or mechanical, can be extremely valuable for time-lapse applications and requires a motorized focus.

Most microscopes designed for live cell imaging sit on a vibration isolation table. Isolation from mechanical disturbance is essential for micro-manipulation, but more importantly, it greatly improves image resolution. Vibration from centrifuges, elevators, autoclaves, and the compressors in freezers and refrigerators blurs the image as it is recorded.

The objective lens

The most important tool for your work is the objective lens. Lens selection begins with identifying three parameters: the numerical aperture (NA), the working distance and the magnification. The NA is the most valuable feature because it determines the lateral and axial resolution limits and the amount of light collected (i.e. the available resolution and contrast). Objective NA ranges from 0.1 up to 1.4, or slightly beyond, where higher NA reflects higher resolving power. High-NA lenses (>1.0) have a short working distance, or maximum focal distance from the coverslip, and require an immersion medium, such as water or oil, between lens and coverglass. Working distance is often overlooked in lens selection, but becomes very important for plant work due to the thickness of live specimens. Magnification determines how large the specimen will appear, but might better be thought of as determining how much of the specimen area will be viewable in the microscope. Generally, magnification increases with NA and decreases with greater working distance.

Microscopes designed since the mid-1990s use infinity-corrected optics, replacing the fixed path length for older objective lenses. The infinity-corrected lenses generally have better light transmission, more working distance, and are not compatible with older microscopes because of the requirement for a tube lens elsewhere in the microscope stand. Objective lenses are corrected to focus red and green (achromat) or red, green and blue (apochromat) to the same image plane. In an achromat, and even some apochromat lenses, the axial focus position for blue and ultra-violet light is different from green and red light, even in lenses where differently colored objects co-align in the lateral field. This causes serious problems when determining the relative positions or intensities of two probes or when ratio-imaging. In addition to chromatic correction, spherical aberrations in the lens can be corrected to achieve a flat visual field (plan).

Microscope manufacturers supply a variety of lenses with nearly the same NA, magnification and working distance. Generally, these lens designs trade light transmission efficiency for better correction or preservation of light polarization. The additional glass elements needed for correcting chromatic and spherical aberrations tend to lower transmission efficiency. Lenses made for fluorescence microscopy (e.g. Fluar, Fluor) transmit more light over a broader spectral range than other lenses, but are often poorly corrected for spherical and chromatic aberration. The absolute light transmission efficiency of a lens tends to be a closely guarded secret. Lenses made for DIC and polarized light microscopy may be highly corrected, but less efficient for light transmission. Polarized light objectives use strain-free glass components that preserve light polarization. Phase contrast objectives have a dark ring inside that only has a slight effect on transmission efficiency. The standard or fluorescence lenses are the best choices for live-plant work, unless high-resolution DIC or polarization is required for the experiment.

Objective lenses are designed to work with coverslips of a specific thickness, usually marked on the side of the objective lens. Nearly all microscopes made for the life sciences have lenses corrected for a number 1.5 coverslip. Using a number 1 or 2 coverslip with these objective lenses markedly degrades image quality. Lenses originally designed for electrophysiology studies, often called dipping lenses, do not require a coverslip at all. These lenses have a ceramic head and dip directly into the bath medium while working with a live specimen.

Oil immersion lenses are designed for imaging specimens tightly pressed to the coverslip. If liquid medium appears between the specimen and coverslip, or if imaging through more than a 20 μm of cell volume, the light-focusing properties of the oil immersion lens begin to falter, and in the worst case, add noise (improperly focused light) to the image. This is very often the case for live plant specimens. Water and glycerin immersion lenses were developed for these conditions because the immersion medium better matches the imaging medium and cytosol. Even though the NA will only reach about 1.2–1.3, instead of 1.4 for oil lenses, the SNR and resolution for the image may be significantly improved under live-cell imaging conditions. Flat-field correction is sometimes poor, but a typical confocal microscope or imaging camera only captures less than the middle two-thirds of the field of view, where most lenses have a flat field, regardless of correction. If the culture medium contains enough high molecular weight material to alter the refractive index, try using the medium between the coverslip and water immersion lens, adjusting the correction collar as explained below.

Prioritize the selection of the objective lens in the following way. Find the highest NA lens that still permits adequate working distance for focusing through the features of interest in the specimen. Choose the lens magnification that will project only the part of the specimen you want to image into the detector. Matching the magnification to the detector will be discussed below. If the specimen is in direct contact with the coverslip, an oil immersion lens will probably provide the best light collection ability. If there is liquid between the specimen and coverslip, or if you are focusing through a lot of cell volume, both the conventional and 'dipping' water immersion lenses may offer better contrast, and near comparable resolution. If the objective lens has a correction collar for adjusting to different coverslip thicknesses and immersion media, it is imperative that you determine the optimum setting (explained below). Microscopy applications requiring short (<380 nm) or long wavelength excitation (infra-red in two-photon excitation) or polarized light require specialized objective lenses where the optical materials will not interfere with the illumination energy.

The illumination system

The illumination system contains a light source, shutters for exposure timing, and filters for selecting both light intensity and wavelength. The goal for specimen illumination is to generate a temporally constant, spatially uniform field of known spectrum for a precise amount of time. Light sources for brightfield microscopy include Tungsten/halogen lamps and light-emitting diodes (LED). Newly introduced LED sources provide a more constant spectrum over their intensity range and generate less heat. Fluorescence microscopy requires more intense illumination, typically provided by plasma arc lamps or a scanned laser beam. Plasma arc lamps, with mercury or xenon as the vapor source, yield enough light that a narrow wavelength band can be selected with a filter for fluorescence excitation. The output from a xenon source is relatively stable and contains a smooth spectrum of wavelengths, both important for quantitative applications such as ratio imaging (see below). Mercury arc lamps are very intense, but show flicker as they age and have an irregular spectrum containing sharp output peaks (see Cyr *et al.*, 2006).

Laser light has special properties harnessed for specific applications. A laser produces one or more monochromatic outputs, often called lines, concentrated in a small diameter (usually 0.5–3 mm) beam. The majority of lasers in imaging systems are either gas filled tubes (e.g. krypton/argon or helium/neon mixtures) or solid-state diodes. Diode lasers have a potentially longer lifetime with less upkeep, although the power and beam quality are often worse than for gas tube lasers. Laser beams remain collimated over long distances, fanning out less over distance than light collected from a lamp and collimated with a lens. Laser power is specified in watts (J sec^{-1}) where specimen illumination is typically in microwatts. The power can be propagated as a

continuous wave or concentrated in time as pulses. The stable, well-focused light from a continuous wave laser is an excellent excitation source when scanned in the confocal microscope. However, lasers are rarely used for widefield fluorescence for several reasons. Expanding the beam to fill the field of view reduces the intensity to below what is required for fluorescence excitation, unless you have an extremely high-powered (expensive) laser. As well, the coherent (in-phase) light from strong lasers imposes limitations on diffraction and interferes with itself in a microscope setting, resulting in randomly speckled illumination patterns. Pulsed lasers carry a tremendous amount of peak power, and are used for such applications as multi-photon excitation and fluorescence lifetime imaging (covered below).

Microscopes developed for live cell imaging need shutters for the illumination system to limit specimen exposure. Mechanically operated shutters, precisely timed to the exposure, are the best tools for limiting specimen illumination time. Be aware that some microscope systems that have not been optimized for live cell work will inadvertently leave the shutter open until the image has been transferred from the detector to a computer. An additional 0.5 sec of illumination time per exposure can be disastrous for time-lapse experiments.

Filters condition the illumination for light frequency (wavelength/color) and for intensity before reaching the specimen. The intensity of non-laser-based illumination should be handled with optical density filters, where possible, and not through changing the electrical power driving the lamp. Optical density filters are typically metal-coated glass or quartz that attenuate the light without dramatically altering the color spectrum. White balance filters, excellent for photographic films, are less useful for digital applications, but green band-pass filters for brightfield illumination will improve resolution by limiting the effects of chromatic aberration in the lenses.

For live cell work, the most critical filter is an infra-red or 'heat cut' filter installed between the lamp and the specimen in both brightfield and fluorescence illumination pathways. Mercury, xenon and halogen lamps produce enough infrared to damage the specimen, and, in many cases, the infrared is not blocked by the fluorescence filter cube. Laser illumination for confocal microscopy does not require this filter, but locating the specimen using unfiltered brightfield or fluorescence illumination on the same microscope can damage the specimen.

Filters for selecting a wavelength or bandwidth of light for fluorescence excitation are most often made of dichroic material and are mounted in a filter wheel or directly into a filter cube. Illumination at any wavelength in an excitation spectrum results in emission over the entire emission spectrum. Therefore, a narrow (approximately 10 nm) band-pass excitation filter is strongly recommended for live cell imaging in plant cells to reduce the background autofluorescence and limit excitation of endogenous fluorophores and sensory molecules. The wide-band-pass filters and short-pass filters in the excitation path of many pre-packaged filter cubes were not designed for plant work because they increase the background fluorescence.

Filters and dichroic mirrors for selecting emission bandwidth are often overlooked when trying to improve the SNR in the imaging system. The dichroic mirror need only have a sharp cut-off between the excitation and emission peaks and minimal attenuation of transmission in the spectral region of interest. Selecting an emission filter requires more thought for both widefield and confocal applications. As the filter encompasses more of the emission spectrum for the fluorophore of interest, the signal will increase for that fluorophore. The signal will also increase for background fluorophores, often crippling the SNR for the experiment. Selecting the correct emission filter requires finding an acceptable balance. If it is possible to obtain an emission spectrum from your sample, with and without the introduced fluorophore, it is valuable in selecting the appropriate band pass filter. Independent of passed bandwidth, the transmission efficiency should be as close to 100% for the entire selected range as possible.

Special filter sets having multiple pass bands, for two- or three-color applications, do not pass as much light as single-pass filters made for the same wavelengths. In order to separate the wavelengths on the monochrome detector, one or more of the light paths will have to be handled separately in the system. You will collect more light by having independent, single-pass filters for excitation and emission, arranged in filter wheels, but this will be traded against the increased likelihood of image misalignment and independent flat field corrections for the two colors. Choosing the best method for multi-color imaging will depend upon the experimental requirements.

The detector

Live cell imaging relies heavily on electronic array detectors, such as CCD cameras, and high-gain point source detectors, such as photo-multiplier tubes (PMT). Although differing in detail, both the digital camera and PMT are analog sensors built on a common principle. Photons impinging on a substrate are converted to electrons and read out as an analog current or voltage stream. Digitizing the signal, by serially approximating the number of electrons in the stream at specific times, creates a relative measure of light intensity, free from the progressive accumulation of noise inherent in storing analog data. By correlating the sampling time with the position of the array element or the scanned beam, the intensity values are spatially correlated to recreate the image. User-definable

detector settings typically include the exposure time, read-out rate, gain and offset levels, and delimiting a portion of the array or scan area for read-out.

Signal generation occurs when photons strike the substrate material on the detector. The exposure time and illumination intensity determine the number of photons generated for image formation. The efficiency of photon to electron conversion is governed by the fraction of the detector surface that can sense photons, called the fill factor, and the substrate's physical quantum efficiency. The detector material inevitably generates a number of spurious electrons, ominously termed 'dark current'. Cooling the detector material, often to –30 or –70°C, suppresses the dark current.

The signal and dark current electrons are read out to an analog to digital converter (ADC). The faster the read-out rate, the higher the 'read-out noise,' either from thermally generated electrons or from small timing errors. Together, the dark current and read-out noise constitute the principle noise sources in the image recording process, beyond the statistical nature of photon counting (shot noise). Many CCD cameras permit selection of a read-out amplifier speed, specified in Hz (bits per second), trading faster frame rates for a SNR loss. Similarly, the scanning speed of the beam, relative to the sampling time of the PMT, can sometimes be changed to increase the signal being read out to the ADC in confocal scanning microscopy.

The ADC samples the sensor read-out over a tightly controlled time interval, synchronized with the read-out amplifier. This is where the photoelectrons and noise electrons from the detector are converted to a gray level or intensity value. The ADC has an input range that limits the total number of electrons before saturation (white), and a set number of electrons that are required for creating a gray level. Dividing the input range by the number of electrons used per gray level yields the system dynamic range, typically specified in bits (i.e. 12 bits $= 2^{12}$ gray levels $=$ 4096:1 SNR $= \sim$72 decibels). A detector with $\pm$4 electrons system noise, probably uses at least four electrons for each gray level and would require an input range of (4×2^{12}), i.e. 16 384, electrons to be digitized to 12-bit resolution. Note that for some sensors with 12-bit (4096), 14-bit (16 384) or even 16-bit (65 500) ADCs, the image detector material may not produce enough electrons to fill the entire ADC input range under all circumstances. The importance of having the ADC bit depth is to capture real differences in signal intensity (i.e. contrast), even if it is captured within a smaller number of gray levels than are available to the sensor.

Setting the gain and offset parameters, together with the read-out amplifiers, allows you to match the incoming signal to the input range of the ADC (see Sluder and Hinchcliffe, 1998). For example, let us say that two adjacent array elements collected 101 and 109 electrons, respectively. If the ADC has an 8-bit dynamic range, and an input range of 2560 electrons, then 10 electrons are required for each gray level. With no electronic gain, the adjacent values would be converted to 10 and 10 for the image. If the detector noise happened to be only $\pm$2 electrons, we might expect that counts of 101 and 109 should be differentiable. Applying a gain of 5, a multiplicative process, yields 505 and 545 electrons respectively, now sampled to values of 50 and 54 by the ADC for the image. Thus, the gain amplifier has increased the difference in detector value such that it is captured by the ADC. As a gain factor of 5 would push any detector value greater than approximately 500 out of the ADC input range, the offset control can be used to subtract electrons prior to ADC entry. Subtracting 400 electrons in the example above leaves 105 and 145 electrons, sampled to values of 10 and 14 in the resulting image. In practice, the gain amplifier introduces noise in proportion to the gain factor, limiting the effective multiplier.

Set the detector to collect light from the smallest possible area of the specimen that still contains the information required for feature identification and context. Imaging a larger area increases specimen exposure to illumination and lengthens the image read-out time. For CCD cameras, read-out area is set in the software controlling the camera. Matching the resolution of the lens to the sensor resolution also needs to be taken into account and will be discussed later. Scanning microscopies also allow software-selectable areas for image acquisition, cleverly disguised as a zoom function. Be acutely aware of the interplay between the zoom and the spatial sampling rate, often recalculated by the microscope software. If selecting a smaller area to image does not change the number of samples (pixels) proportionately, then the goal of shortening the illumination and read-out time has not been achieved. You must reset the image size with the sampling rate, keeping in mind the resolution limits of the lens discussed below.

Implementations of modern image detectors

While the same basic principles apply to most detectors, implementations differ markedly. The key design feature of the CCD is that it stores electrons during exposure, without building up substantial noise, providing exceptional dynamic range (Berland *et al.*, 1998). The exposure time and read-out rate are the critical parameters. CCD sensor elements act like photoelectron collecting buckets (Aikens *et al.*, 1989), starting with zero electrons and filling to a defined saturation point, termed the electron well depth. For most CCD cameras, the well depth is matched to the ADC input range, precluding the need for user-controllable gain and offset parameters. Selectable read-out amplifier speeds allow trade-offs between SNR and maximum frame rate. The other virtues of the CCD are the high quantum efficiency, up

to approximately 90% in some cameras, and excellent response linearity, where the output signal is tightly correlated with photon counts over the entire dynamic range. The advantages of the CCD camera are exploited in three ways. First, long exposures can be used to collect light from a weak signal source for detection. Second, the dynamic range permits local contrast differences within bright and dim features to be captured in the same image. Finally, the response linearity simplifies the background subtraction and calculations required for quantitative applications.

Traditionally, a 'video' camera denoted a vacuum tube camera, and a 'digital' or 'scientific' camera referred to a CCD device. While most video cameras now use solid-state chips, an important difference persists with the jargon. Typical video cameras, engineered to output 30 frames per second, sacrifice sensitivity and resolution for cost and speed. A video camera can be used for brightfield work, but quantifying relative differences in intensity may be stymied by auto-gain or gamma-correction circuits, and, in some cases, the image suffers from geometrical distortions due to non-square pixel dimensions.

Color CCD cameras collect the red, green and blue (RGB) parts of the spectrum into separate images and blend them after exposure to simulate color vision. Video rate cameras use a color mask printed over the CCD chip, lowering the resolution by a factor >3 for even the best cameras. Better color cameras either take three consecutive images, using a filter wheel to select color bandwidth, or have three CCD chips and a means for dividing the incoming light into red, green and blue for simultaneous acquisition. Except in very specialized circumstances, the lack of acquisition speed, sensitivity, dynamic range and resolution for a color camera make imaging live cells extremely difficult. Notable exceptions are newly designed multi-chip cameras used in ratio-imaging and FRET applications (Nagai *et al.*, 2004).

The PMT, and related devices, have mediocre quantum efficiency, up to 40% in the best cases, and generate a large number of spurious electrons (noise) compared to a CCD (Sandison *et al.*, 1994). The PMT does not typically store charge, but rather uses high gain and variable offset controls to boost signal above noise. The principal advantage of the PMT is its ability to measure the rapidly changing intensity of a point source, such as the specimen fluorescence resulting from a scanned laser beam. For each successful photoelectron conversion in the substrate, the PMT amplifies the signal into many hundreds or thousands of electrons before ADC sampling. Rapid sampling, usually on a microsecond scale, lessens the effect of dark current build-up, and applying a massive gain to the signal before read-out reduces the impact of read-out noise. Therefore, signal to noise ratios for PMT systems are typically dominated by the photon counting variance, the 'shot' noise, previously described. Taking advantage of the gain and offset control for a PMT allows extraction of meaningful dynamic range from laser scanning confocal and multi-photon systems.

There are many other detectors that you may encounter. Cameras using complementary metal oxide semiconductor (CMOS) sensors have displaced many CCD technologies in the consumer market, but the poor quantum efficiency (approximately 25%) presently limits usefulness in low-light applications for scientific work. Intensified CCD cameras (ICCD) have an array of micro-PMTs, called a microchannel plate (MCP), usually fused to the CCD chip. The MCP limits spatial resolution and response linearity, but provides excellent signal detection capabilities when high frame rates are critical (Colarusso and Spring, 2003). Electron-multiplying CCD cameras (EM-CCD) have a gain amplifier built into the CCD chip, allowing multiplication of the signal electrons prior to the addition of read-out noise. These cameras provide all of the fundamental virtues of a standard scientific CCD, with the additional property that fewer photoelectron conversions need to take place before the ADC can convert the signal to a usable dynamic range. Solid-state alternatives to the PMT have been used for many years and include specialized CCD units and avalanche photodiodes (APD).

4. Microscopy techniques

The live plant cell is a thick specimen. Out of focus information blurs the image and the constant churning of the cytoplasm places limitations on exposure times. Both brightfield and fluorescence methods used in imaging plant cells must take into account the sensitivity of plants to light exposure and the problems associated with resolving features more than 20–30 μm within the specimen. Brightfield techniques are often less harmful to live cells, but methods for observing specific proteins using trans-illumination have not been widely developed for live cells. Generating a high-contrast chromatic (color) or intensity difference in a brightfield image is more difficult than identifying a luminous intensity change (e.g. fluorescence) against a dark background. Therefore, brightfield methods find use in following organelles or cell-wide behaviors, and fluorescence methods, including confocal techniques, are used for following specific molecules.

Brightfield microscopy

Brightfield microscopy relies upon changes in light absorption, refractive index or color for generating contrast (see Inoue, 1986). As light passes through the specimen, areas that alter the direction, speed or spectrum of light generate optical disparities (contrast) when the light is collected and focused by the objective lens. Resolution in a brightfield system depends on both the objective and condenser NA, and an immersion medium is often required on both sides of the specimen. CCD cameras provide the wide dynamic range

and spatial resolution required to capture the information present in a brightfield image. Background subtraction, using averaged frames taken with no specimen in the light path, increases contrast dramatically (Salmon *et al.*, 1989).

Simple brightfield viewing, with properly adjusted Köhler illumination (see below), provides information about the cell outline, nuclear position and vacuolar boundary. Methods that enhance contrast include differential interference contrast (Smith or Nomarski DIC), polarized light, phase contrast, Hoffman modulation contrast and darkfield microscopy. Specimen thickness and the optical activity (bi-refringence) of the cell wall limit the use of these techniques in plants.

DIC microscopy uses plane-polarized light and additional light-shearing prisms to exaggerate small differences in specimen refractive index. Lipid bilayers, for example, show up well in DIC because of the difference in refractive index between aqueous and lipid phases of the cell. The bi-refringent cell wall reduces the contrast-generating power of the technique, but a properly aligned system should permit visualization of nuclear and vacuolar membranes, some mitochondria, chloroplasts and condensed chromosomes in epidermal cells. DIC is an important technique for plant work because, in addition to the increased contrast, DIC decreases the depth of focus, creating a thin optical section of the thick specimen.

Polarized light microscopy is achieved by viewing the specimen between crossed polarizing elements. Structures within the cell with bi-refringent crystalline properties, such as cell wall and starch granules, rotate the plane of light polarization, appearing bright on a dark background. Phase-contrast and Hoffman modulation will increase contrast for plant protoplast and tissue culture cells, but typically result in excessive 'ringing' around the cells in plant tissues. Darkfield microscopy is also limited in use to physically isolated cells. In darkfield microscopy, the condenser directs light into the specimen at a steep angle, so that it misses the objective lens, unless redirected by the specimen, resulting in a dark background with bright specimen detail. The thickness of plant tissues generally redirects most of the light into the objective path, reducing the effectiveness of the technique.

Widefield fluorescence microscopy

Widefield fluorescence imaging with a CCD camera and shutters constitutes the simplest form of live cell fluoresence work (see Stephens and Allan, 2003). Fluorescence microscopy uses epi-illumination (through the objective) and relies upon interference filters for selecting a bandwidth of emission for detection (see Taylor and Salmon, 1989). The full-aperture light collection maximizes recorded signal and minimizes exposure time. The main drawback to widefield imaging is that fluorescent objects and background, out of the principle focal plane, contribute unwanted light that obscures the feature of interest. Therefore, widefield imaging works best when the feature of interest is large, like an organelle, or punctate in nature. If the feature is easily resolved, widefield is the best method for live cell work.

Computational methods for reducing the effect of out of focus light, often called deconvolution or deblurring, work better for fixed specimens than for live cells. The computations work best when used on a series of images taken from above to below the feature of interest. The algorithms require a high signal count (long exposure) and an optically homogenous medium, often hard to achieve when imaging live cells. Other commercially available methods using structured illumination, usually a grid pattern, for producing pseudo-confocal images suffer for the same reasons when applied to living plant cells. Multiple, high dynamic range images are required for the computations, slowing acquisition time and often resulting in excessive photodamage to the specimen.

TIRF fluorescence microscopy

Total internal reflection (TIRF) microscopy requires that light, usually from a laser source, enter the coverslip at a shallow enough angle that it reflects off the internal glass surfaces without exiting to the specimen (Axelrod *et al.*, 1983). This total internal reflection within the coverslip creates an evanescent surface wave with enough power to excite fluorophores within about 50–100 nm of the coverslip surface, dependent upon laser power. The extreme limitation on excitation depth works wonders for single molecules on coverslips or for adherent mammalian cells but shows little promise for work on plant cells that typically have cell walls >100 nm thick (frustrating personal observation).

Scanning fluorescence microscopy

For scanning microscopy, a focused beam restricts the illumination area, limiting excitation to a spot about the size of the objective lens resolution limit. The resulting fluorescence emission returning to the detector can be spatially filtered, using a confocal 'pinhole', to capture only the center of the emitting spot. As the pinhole aperture is reduced, it blocks more out of focus light from being detected but also lowers the total signal (Sandison *et al.*, 1994). While the absolute signal value is less than for the widefield microscope, rejecting the out of focus light increases the specific SNR for the feature of interest. Most strikingly, the contribution from above and below the plane of focus drops off markedly, providing the ability to optically section the specimen. For thick plant cells, this capability makes scanning confocal microscopy invaluable for imaging fluorescently labeled structures. Multiple detectors are easily integrated

into scanning microscopes, making it possible to simultaneously, as opposed to sequentially, image multiple fluorescent colors.

The major disadvantage of conventional laser scanning microscopy for imaging live plant cells is that the image acquisition time is relatively slow, often several seconds per image. There is also a tendency to over-irradiate the specimen. The dwell time of the beam at any sample position may be only a few microseconds. The excitation energy must be high enough to generate usable signal during that dwell time, often leading to complete saturation of all fluorophores in the spot. Under these conditions, photobleaching occurs, and collecting images with a high intra-scenic dynamic range (lots of gray levels) is challenging.

Multi-photon excitation microscopy, also called 'two-photon' microscopy, uses a scanned beam of pulsed laser light. If two photons of twice the excitation wavelength hit the fluorophore, in the right time interval, a single excitation event occurs. The push to develop multi-photon microscopy stems from the following ideas. As most specimens are transparent to infra-red light, excitation should be less damaging to the biological specimen and should penetrate deeper into tissues, providing extended imaging depth with less phototoxicity (see Stutzmann and Parker, 2005). Further, since the probability of excitation is related to the square of the illumination intensity, excitation occurs only in a very small focal volume. The small excitation volume relaxes the requirement for the spatial 'pinhole' filtering of the emission used in confocal microscopy, and thus more signal is theoretically available from two-photon excitation than from conventional confocal microscopy. The technique shows great promise for plant systems, although it has proven to be problem-laden in the initial commercial applications (Feijo and Moreno, 2004). Two-photon scanning microscopy has been used in living plants to image weakly fluorescent features, several cell layers into the specimen (Nakajima *et al.*, 2001). Successful application of the technique requires access, often difficult given the expense and expertise required to run the pulsed laser source, and practice in finding the correct wavelength and power range to excite the specimen without rapidly photobleaching the fluorophore.

Hybrid scanning techniques include confocal spinning disk microscopy and line-scan confocal microscopy. Both techniques were developed primarily for live cell imaging where an increase in image acquisition speed is gained for some loss of axial resolution. The spinning (Nipkow) disk has spirally arranged pinholes (see Maddox *et al.*, 2003) that scan the entire image, acting as a multi-point confocal system. The image is recorded using a CCD camera, not a PMT, so using a spinning disk is similar to simple widefield fluorescence imaging. The disadvantage of the spinning disk is that the pinhole size is typically fixed, so the degree of confocality is no longer user-controlled, and only a subset of objective lenses (numerical apertures) will work well with the disk (see Egner *et al.*, 2002). When used with an EM-CCD or ICCD camera, spinning disk systems are proving to be very important tools for live cell work.

Confocal systems using a line to scan the specimen, instead of a point, decrease the image acquisition time at some expense to resolution. Unlike the spinning disk, the degree of confocality can be user-controlled. Instead of a PMT, line-scan confocals use a linear CCD element to record intensity. Newly introduced line-scanning confocal devices will be extremely useful in imaging the rapidly changing cytosolic compartments within plant cells.

Special techniques in fluorescence imaging

Fluorescent molecules have several important properties that can be measured to characterized biological activity or the cellular environment. Fluorophores have a probability of capturing a photon (extinction coefficient) and an associated probability of releasing the energy as a photon (quantum efficiency) or in some other form. The probability of accepting a photon occurs over a limited bandwidth of excitation energy (excitation spectrum) and the emission is restricted to a lower energy, longer wavelength bandwidth (emission spectrum). Losing the excited state electron (photobleaching) blocks the fluorescent emission and neuters the fluorophore except under special circumstances in some fluorescent proteins (Lippincott-Schwartz and Patterson, 2003). The time between photon capture and fluorescence emission is random, showing an exponential relationship with a characteristic half-time, specific for the fluorophore (fluorescence lifetime). If the excitation energy is plane-polarized, the emission will also be plane-polarized, with the degree of emission polarization determined by the amount of rotation the molecule underwent in the time interval between accepting the excitation energy and emitting the fluorescent photon. Each of the described properties of the fluorophore is highly dependent upon the local (solvent) environment. Small changes in ion concentration, proximity to electron donors and acceptors, and even solvent viscosity can have dramatic effects on fluorescence intensity and lifetime. Creating fluorescent probes that are sensitive to specific changes in the environment has allowed the biologist to monitor *in vivo* events in real time.

Ratio-imaging intracellular intensity changes

Fluorescence ratio imaging was developed to quantitatively assess changes in molecular environment within the cell (see Gilroy, 1997). Ratio imaging deals with the inherent problem of determining when an intensity difference is due to a change in probe property and not a change in probe distribution (see Dunn and Maxfield, 1998). Ratio imaging works by having a probe that can be measured at two

wavelengths, where at least one wavelength is sensitive to the environmental change you want to investigate. When, for example, calcium ions bind to certain dyes, the primary excitation peak shifts by >30 nm. Therefore, as calcium binds, the apparent intensity of the dye decreases. If a second image is taken, exciting the dye at the new maximum excitation wavelength, the dye intensity appears to increase with increasing calcium ion concentration. By dividing one image by the other, the intensity changes become normalized to the amount of dye at that position in the cell. In other words, the change in intensity can be attributed to a change in dye property and not dye distribution. Further, the amount of ratio change can be calibrated to the change in calcium ion concentration over a physiological range, so the fluorescence ratio can be converted to an intracellular calcium ion concentration. If the environmental change alters the emission or excitation spectrum of a single probe, the gain in intensity in one channel is exaggerated by a loss of intensity in the other. If using two probes, one sensitive and one insensitive to the cellular change, it is advantageous to have them coupled to a single carrier molecule, ensuring that they represent exactly the same cellular volume.

Ratio imaging can be accomplished using two excitation wavelengths, two emission wavelengths, or a combination thereof, using confocal or widefield systems. New optical instruments for simultaneously recording two-color channels on two sides of a CCD detector have increased ratio acquisition time for dual emission probes in widefield systems (Fehr *et al.*, 2004). As well, some confocal and multi-photon systems have a detector device that measures a spectrum of emission wavelengths (Berg, 2004), greatly improving the accuracy of the ratio measurements. Regardless of equipment, quantitative ratio imaging is a perilous undertaking for the plant cell biologist. Getting non-genetically encoded probes into the cell often results in cell damage from injection, retention of dye in the cell wall, or sequestration of dyes over time into unwanted places. Cytoplasmic streaming rapidly mixes the probe, making the acquisition of high dynamic range sequential images very challenging. Patterned and time-dependent autofluorescence is nearly always present unequally in the ratio pair. Finally, *in vivo* calibration for ion or membrane potential measurements can be extremely frustrating given the difficulty in introducing compounds into the cytoplasm that are not influenced by the cell wall.

Advanced fluorescence techniques

FRET imaging uses the same equipment required for quantitative ratio imaging, with special attention paid to the controls (Bhat *et al.*, 2005; Hink *et al.*, 2002). Non-radiative energy transfer occurs between a donor and acceptor molecule if they are spatially aligned and within a few nanometers of each other. By excitation of the donor molecule and image capture of both donor and acceptor emission, some account of the degree of energy transfer can be taken, either through determining the amount of quenching for the donor or the intensity from the acceptor. For example, a protein labeled with cyan fluorescent protein (CFP) yields a blue emission that will be lessened if a second protein, fused to yellow fluorescent protein (YFP), binds in such a way that FRET occurs between donor (CFP) and acceptor (YFP). Any non-FRET-related bleedthrough of fluorescence from donor channel to acceptor channel and any non-uniform autofluorescence compromise the FRET result in a non-linear fashion (Berney and Danuser, 2003). When appropriately used, FRET imaging provides an intuitive means for assessing when and where proteins interact in the cell or when a single protein is undergoing a large conformational change.

Another powerful method for assessing changes in protein state involves bleaching the fluorophores within a subdomain of the cell (Weiss, 2004). Provided the bleaching does not damage the associated molecule under study, kinetic analysis of fluorescence recovery provides information about the rates of diffusion or binding within the live cell (Elson and Qian, 1989; Wachsmuth *et al.*, 2003). The idea is that the molecules of interest, attached to the bleached fluorochromes, are replaced through diffusion and through association and dissociation with the pool of molecules having attached, unbleached fluorophores. Fluorescence recovery after photobleaching (FRAP) uses a laser or flashlamp to rapidly bleach a small area, and images are collected at unit time intervals for measuring recovery. As discussed for ratio imaging, the effect of cytoplasmic streaming must be dealt with for cytoplasmic components because the diffusion process is colluded with directional cytoplasmic streaming in the plant cell. Fluorescence loss in photobleaching (FLIP) uses a laser to bleach fluorophores in a cellular compartment while intensity measurements are made in associated compartments to evaluate intracellular connectivity.

An adaptation of the confocal principle has also been employed to estimate diffusion properties and binding affinity within a cell. Fluorescence correlation spectroscopy (FCS) takes a single spot within the cell and rapidly samples the number of photons emitted from the molecules in that spot (Goedhart *et al.*, 1999). A diffusible molecule wandering in and out of the confocal volume will have a characteristic residence time. As diffusion becomes restricted, for example by binding to a surface or larger macromolecule, that time in and out of the volume will change characteristically. An auto-regressive function is applied to the time-resolved intensity data to determine residence times in the confocal volume. Using this principle, confocal FCS permits estimation of diffusion rates and binding constants within the live cell for extremely small numbers of molecules.

A fluorescently labeled protein binding to a larger macromolecular complex in the cell undergoes a state change. As the protein binds, its conformational state and degree of rotational mobility are altered. In some cases, the change in conformational state affects the fluorescence lifetime. Fluorescence lifetime imaging microscopy (FLIM) is accomplished using pulsed lasers with time-resolved photon counting detectors or with phase-resolved techniques (French *et al.*, 1998; Immink *et al.*, 2002). Binding the larger complex may also alter rotational mobility, reflected in a change in fluorescence polarization anisotropy. Polarization anisotropy measurements involve quantitative ratio imaging of polarized excitations using perpendicular and parallel polarizers in the emission path (Axelrod, 1989; Axelrod *et al.*, 1983; see Lidke *et al.*, 2003).

5. Acquiring data with the microscope

Successfully imaging live cells requires a working knowledge of the imaging system. Changing the microscope or the detector settings changes the contrast and resolution. So how do you know if that made the image better or worse? Even if the image improves to your eyes, that doesn't always translate into better data collection. Computer monitors vary and your eyes and brain make adjustments for missing information in an image. In this section, two tools are introduced for determining image quality, the line scan and intensity histogram (Figure 5). The line scan tool plots the intensity information from a transect of the image, allowing you to visualize the relationship of signal to noise. The histogram tool displays image intensity values to graphically illustrate the dynamic range present in the image. Three prepared slides will also be introduced, and their use, in conjunction with the software tools, to align and calibrate the microscope for brightfield and fluorescence observations, will be described.

Alignment for brightfield microscopy

Nearly all modern microscopes are designed for Köhler illumination (Keller, 1998), maximizing resolution and contrast through incoherent illumination with collimated light rays. Place a slide on the stage and focus on the specimen using a low power (10–20×) objective lens. Aligning for Köhler illumination requires closing down the field aperture between the condenser and lamp, using the condenser to bring that aperture into focus, and then centering the aperture with the condenser centering screws. When the iris is opened to just fill the field of view with light, the microscope is aligned for brightfield work. This alignment procedure works because the microscope manufacturer placed the aperture (field diaphragm iris) so that it is in focus when the lamp is in focus. Re-align the microscope for each lens before taking images. Note that a light diffuser, such as a ground glass filter, must be placed in front of the lamp to evenly illuminate the field.

The line scan tool, found in nearly all microscopy-related software packages, can now be used with a stage micrometer slide to calibrate the pixels per micron and determine the sampling rate of the detector. Place the micrometer slide on the stage and, if required, apply an immersion medium between coverslip and lens. Focus on the lines and take an image using the camera (or reflected light and the PMT for confocal systems). Using the line scan tool, draw a line over several of the stage micrometer lines with the mouse and create an intensity graph. The graph should show the intensity value at every pixel position under the line. Divide the number of pixels counted from the center of one black bar to the other into the real bar distance (in micrometers) to calculate the pixels per micron. Repeat the process several times, moving or rotating the slide before each exposure. If the software counts pixels, and not distance in pixel units, you may find that the distance measured when the slide is 45° to the detector array is less than when the slide is 0° or 90°. This is obviously incorrect and the Cartesian (square grid) nature of the distance measurements must be accounted for when calibrating the system and using the software. If there is an intermediate lens (e.g. optivar), increasing the magnification by 1.5–2.5×, repeat the above procedure to ensure the stated magnification change occurs precisely as advertised. The calculated pixels per micron can now be used for distance measurements and for determining how much of the lens resolution is captured by the detector.

Matching the detector sampling rate with lens resolution, specified by the lens NA, involves a little mathematics and adjusting the magnification to the detector. The commonly accepted theoretical resolution limit for a lens, the Rayleigh criterion, is calculated as (1.22 × light wavelength)/(objective NA + condensor NA) for brightfield systems and (1.22 × light wavelength)/(objective NA × 2) for epifluorescence and confocal systems. For epifluorescence microscopy, the objective lens counts twice because it serves both as condenser and collecting lens (see Parker, 2003). As a suggested starting place, each resolution unit distance should be covered by at least three and no more than eight pixels. If the lens image is under-sampled in a camera-based system, it can be magnified using the intermediate lens or by changing the projection lens to the detector, located in the camera port, for one of higher power. If more than eight pixels span the resolution unit, the CCD elements can usually be blocked together, or binned, 2 × 2 as they are being read out. Alternatively, a projection lens of lower power can be used. For scanning microscopies that use a PMT or similar device, the spatial sampling rate is determined by the beam scan rate, the imaged area, and the number of samples per distance. For example, scanning a

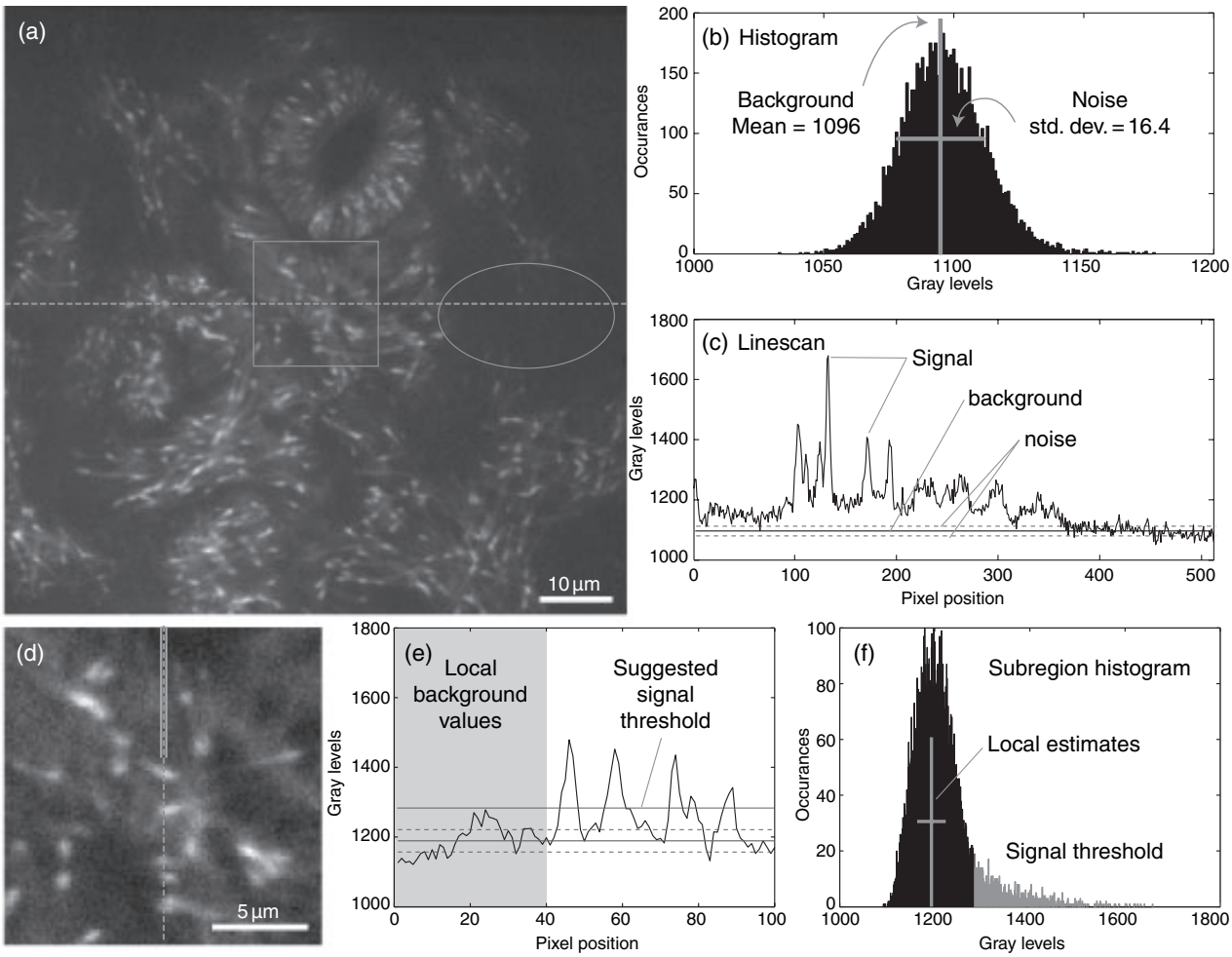

Figure 5. Histogram and line scan tools for determining background, noise and signal levels.
(a) One image from a time-lapsed series of live *Arabidopsis* cells expressing a GFP fusion protein (*AtEb1*-GFP) labeling polymerizing microtubule ends. The image was taken with a 63 × 1.2 NA water-immersion objective lens and spinning disk confocal unit using a 512 × 512 pixel EM-CCD camera.
(b) Global background and noise estimates were made using a histogram of the pixel values from a featureless region (ellipse) in the image. The mean value estimates background and the standard deviation estimates noise.
(c) A line scan across the image (dashed line in (a)) shows the pixel values at each position in the image. The estimated background (solid line) and noise (dashed lines) are plotted with reference to the pixel values. Note that the plotted background value fits the line scan values for the right side of the image, but the background in other regions of the image appears higher.
(d) To better approximate image properties for features of interest, a local estimate of background and noise is made from a sub-region of the image [corresponding to box in (a)].
(e) A line scan [from dashed line in (d)] shows the pixel values for several of the fluorescent features of interest and for a region containing no features of interest [gray bar in (d), gray background in (e)]. Local background and noise estimates, calculated as the mean and standard deviation of the first 40 pixels in the line scan, are plotted in (e) with reference to the pixel values. In addition, a suggested minimum signal threshold value is plotted where signal exceeds background by >2.7 times the magnitude of the noise.
(f) The local background estimate approximates the distribution mode for a histogram of the pixel values for the entire image sub-region. Values above the suggested minimum signal threshold (gray portion of histogram) are found in the distribution tail. Creating local estimates for signal, background and noise using the line scan and histogram tools serves as a useful method for determining how to adjust the acquisition parameters at the beginning of an experiment.

100 μm line in 2 msec, taking 2 μsec samples, is equivalent to imaging the same space with 1000 pixels or 10 pixels/μm. Most laser scanning confocal software packages specify permissible scan times that bracket the theoretical resolution limits for the lens. Regardless of system or what the software tells you, work out the pixels per micron empirically with a micrometer slide and be certain that you are not over- or under-sampling the lens resolution.

Micrometer slides serve additionally as brightfield contrast and resolution targets. Provided the bars are opaque, the difference in average values between the white and black regions provides a measurement of dynamic range. Increasing the illumination increases the number of gray levels between black and white until the white portion saturates the detector. Using the line scan tool again, but drawing a line across only two bars, note that the transition from black

to white occurs over several pixels and that the bars are not uniformly dark. Even if the bar has a uniform molecular thickness of opaque material, the physical properties of the condenser and objective lens will blur the transition. For higher NA lenses, closing the condenser iris, not the field diaphragm iris, will alter the transition, even after adjusting for the intensity change. As the iris is closed down, some contrast is gained, while resolution is lost. Partially closing the field diaphragm iris to just surround the image should sharpen the transition from dark to light, because there is now less stray light in the optical system.

The line scan tool is probably the best tool to start with when trying to improve the quality of the image in a live cell experiment (Figure 5). As you are adjusting the optical system and detector controls, the line scan tool should be used to report the effect of your adjustment. Regardless of what a particular monitor might show in the microscope room, the line scan tool does not lie. Drawing a line over the specific feature of interest and plotting the intensity values allows you see how much of the detector dynamic range you are capturing and the separation of signal from background and noise. If the difference between black and white is increasing on the graph, you are increasing contrast. If the transition between black and white is occurring over fewer pixels, you are increasing resolution.

Alignment for fluorescence microscopy

The energy required for fluorescence excitation comes from a small plasma arc or a small-aperture laser device. The illumination pathway for the microscope magnifies the image of that light source and projects it onto the specimen for excitation. While the illumination will never be completely uniform, the goal is to create as uniform an excitation as possible.

Alignment for an arc lamp is a two-step process. The first part is greatly facilitated if you focus on a piece of lens paper or a business card placed on the stage using brightfield trans-illumination. Align the condenser for Köhler illumination and then close the field diaphragm iris down so that the condenser projects a small spot on to the paper. That spot represents the optical axis of the microscope where the fluorescence excitation needs to be projected. Remove the objective lens from the nosepiece and open the shutter to the fluorescence lamp, with fluorescence filters in place. Use the collimating lens on the lamp housing to create an image of the plasma arc on the paper. Most lamp housings have a mirror that creates a second image of the arc. The goal now is to create a focused image of both arcs using the collimating lens and all of the knobs on the back, and possibly the side, of the lamp housing. Knobs for the lamp move both images while moving the mirror only moves one image. Next, position the arcs so that they are overlapping about 25% and centered on the bright spot projected from the condenser. Place a neutral density filter into the fluorescence excitation path in order to see the trans-illuminated spot. Defocusing the arc images with the collimating lens should now allow you to produce an evenly illuminated field on the lens tissue. Close the shutters for both trans- and epi-illumination paths and replace the objective lens to complete the first step.

The second step aligns the lamp with the detector. For widefield and confocal fluorescence illumination, dilute a fluorescent dye matching the excitation/emission spectrum of interest into the imaging medium (500 pM–1 nM final concentration). Centrifuge for 10 min in a microfuge before applying 10 μl to a clean slide and adding a number 1.5 coverslip. Alternatively, plastic fluorescent slides are available from some microscope vendors. Take an image of the slide and create an intensity graph from a diagonal transect using the line scan tool. Typically, the intensity distribution is not centered on the detector and falls to <80% of the maximum at the corners. Continue to take images and monitor the line scan tool graph as you adjust the collimating lens and lamp housing to achieve as uniform a field as possible. The corners may not reach more than 85% of the intensity found in the image center. Some software packages allow you to false-color the image with a pseudo-colored LUT, making it easier to visually center the lamp with the detector. Non-uniformity in point-scanning confocal microscopes typically arises from a misalignment or mis-timing of the scan beam. Save the image to use as a reference for discussing the problem with the service representative.

System noise and response linearity

The dye slide can subsequently be used for estimating system linearity, locating optical noise, characterizing the gain and offset parameters, and for creating an intensity/shading correction image. For CCD cameras, begin by taking exposures of increasing duration (0.01, 0.05, 0.1, 0.5, 1.0, 5, 10 and 50 sec) with no light to the detector. Displaying the intensity values from the 50 sec exposure as a histogram should reveal a bell-shaped curve. The average intensity value, usually the peak of the bell curve, represents a background and the standard deviation represents the uncertainty or detector noise. The average values for the shorter exposures should remain initially constant and begin to increase as the time-dependent dark current becomes significant relative to the read-out noise. Outliers in the distribution will appear as a result of pixels that consistently yield higher than normal values (hot pixels) or from other phenomena such as cosmic rays. If there is a gradient across the image or any other patterned feature, light may be leaking to the camera or the CCD chip may not be cooling correctly. Repeating the exposure process while weakly exciting the dye slide (i.e. using a neutral density filter) yields a measure of system linearity. The average value for each frame should be a linear

function of the exposure time, with linearity improving after subtracting the background values taken from the previous exercise. Changing the gain, offset or the read-out amplifier will change the relationship between exposure time and average value.

For confocal users, exposures with no light to the specimen are less useful in estimating background as dark current should not build up appreciably in the PMT. Understanding response linearity and the role of the gain and offset controls is, however, critical to using the device. Using a fast scan speed and low (<3%) laser power, take several exposures, increasing the gain by a unit interval (try 2% of total allowable) each time. If the offset is used to bring the digitized values close to zero for the first exposure, increasing the gain should increase the average value by a non-linear factor for each subsequent exposure. Further, the noise (i.e. the standard deviation) also changes in magnitude. Keep this principle in mind when comparing the intensity values from confocal images. If the laser power can be incremented in small steps (1–5%), taking exposures with increasing laser power should yield a grossly proportional increase in average signal value, although the relationship will probably not be linear. To estimate the linearity of the system at one exposure setting, where gain, offset, scan speed and area are left constant, the simplest method is to create multiple slides using a serial dilution of the fluorescent dye.

Optical noise in the image

The microscope light path and detector surface are never completely devoid of dust and debris that change the optical path. This optical noise is easily discovered when images are taken from multiple regions of the dye slide. Optical noise appears as local non-uniformities in the image intensity that persist when the slide is moved. This optical noise will locally shade or alter the resolution of features within the image. The major offender is usually the objective lens. Try cleaning the objective lens (Inoue, 1986) with specialized lens tissue (never use other lab wipes or non-optics-related material) and an ammonia-based glass cleaner. Most fluorescence filters and many other optical surfaces have delicate coatings created to reduced reflection. These coatings can be damaged by solvents or mechanical wiping and should be cleaned professionally or with the advice of the manufacturer. Pressurized gas effectively clears dust from optics but extreme care should be used in not pointing the air stream directly at (90° to) the optic or in spraying the optical component with liquid that would result in freezing condensate on the surface.

Multiple images of a dye slide, usually from multiple positions on the slide, can be averaged together, normalized, and divided into an image of the specimen to digitally flatten the field and lessen the effects of optical noise and uneven illumination (Model and Burkhardt, 2001). This shading correction is extremely valuable when performing quantitative tasks such as ratio-imaging or FRET imaging.

The fluorescent bead slide

A fluorescent particle smaller than the resolution limit of the microscope creates a distinct, diffraction-dependent pattern in the microscope. The fluorescent light emitted from a particle of 100 nm diameter flares out over an area larger than expected for the calculated magnification. This point-spread function (PSF) describes the influence of the optical system on a point source of light. The PSF is, in essence, what happens to every point of light emitted from the specimen, and forms the building block for creating the intensity patterns that constitute an image.

Using the idea that light energy travels as waves, a theoretical PSF can be calculated mathematically from the objective NA and light wavelength. The theoretical PSF image is rotationally symmetric, having a strong central peak and several smaller peaks spaced at intervals from the main (Figure 6). The smaller peaks form concentric circles, expanding out from the central peak on both sides of the focal plane. The concentric rings diverge from the focal plane as cones, creating a series of hourglass-shaped patterns of increasing size when observed from the side (Figure 6). The qualities of the PSF depend upon lens type, lens alignment, lens cleanliness, correct coverslip thickness, correct immersion medium, proximity of specimen to coverslip, and uniformity of illumination intensity.

As we know what the PSF should look like in theory, observing how the real PSF differs in practice informs us about the optical system. Simple bead slides, made by dispersing fluorescent latex particles in an imaging medium, make an excellent tool. As plant cells are thick specimens, coating the face of a slide and a coverslip with poly-L-lysine before adding beads results in two spatially distinct layers of immobilized particles when the slide is assembled using a drop of medium. Focusing on well-dispersed beads, the peak intensity should be comparable across the field of view and the central intensity of the beads should appear round and not elliptical. The next test for the bead slide is to see whether concentric rings appear on both sides of the primary focal plane. If not, be absolutely certain that the coverglass number, usually 1.5, matches the number printed on the objective lens. If the lens has a correction collar, use it to balance the distribution of fluorescence above and below the primary focal plane, making the rings as axially symmetric as possible. The correction collar on the objective lens permits compensation for coverslip thickness and for differences in immersion media. If the slide has beads at two levels, try and find a compromise adjustment for

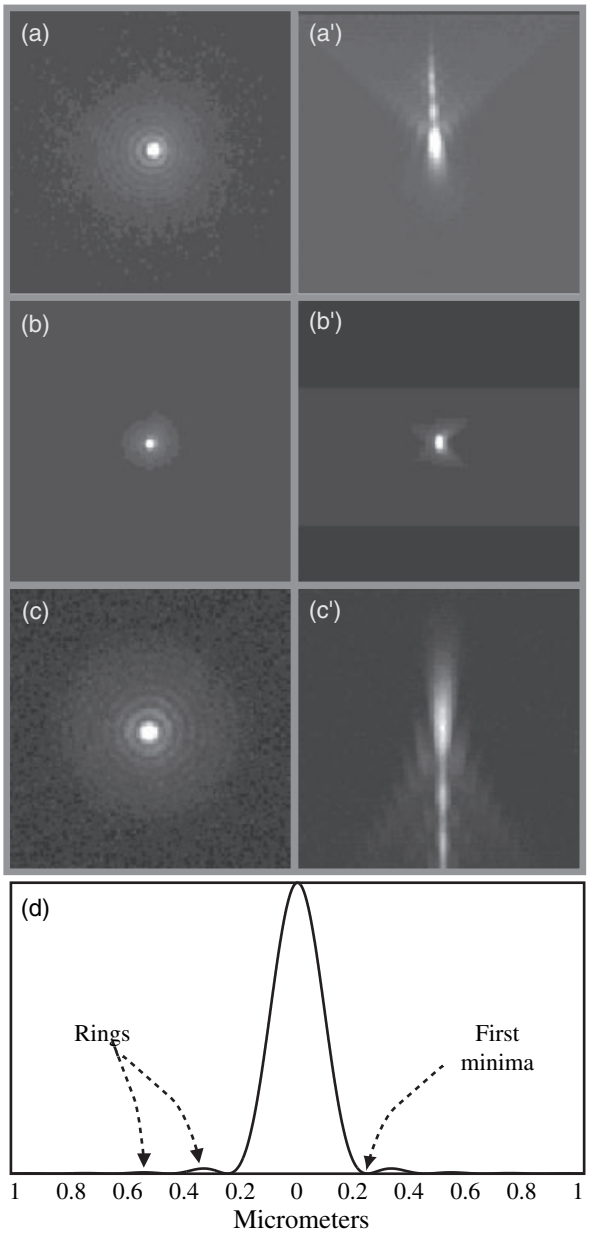

Figure 6. Point spread function for a sub-resolution fluorescent bead.
A 100 μm diameter fluorescent latex bead was imaged with a 0.75 NA objective lens. The correction collar was adjusted from under-corrected (a, a′), to properly corrected (b, b′), to over-corrected (c, c′). A series of images was taken for each setting, focusing through the bead at 0.5 μm intervals. Panels (a–c) are forward projections from the series and panels (a′–c′) are side-on projections of the data. Note the tightly confined spot, both axially and laterally, for the projections in b and b′. The improperly corrected lens yields an axially asymmetric point spread function (a′, c′). (d) The theoretical PSF for a 1.4 NA lens, illustrating the position of the diffraction rings and first minima. The distance between the PSF peak and the first minimum is given by the Rayleigh resolution criterion.

beads at both depths. For lenses without a correction collar, using an immersion medium or bath medium with a different refractive index may be required to correct the problem. If the problem persists, consult your microscope dealer and ask whether the optics require adjustment.

Use double-labeled beads, or create two-color particles by combining cationic and anionic beads of different colors, to verify that the there is no lateral or axial chromatic shift in the microscope. Lateral translation of the bead colors in one direction most often results from misaligned fluorescence filter sets. Axial misalignment is more difficult to remedy because it is generally a property of the chromatic corrections for that lens. Beads in the periphery of the field may have an elliptical shape or have laterally asymmetric rings around the central axis, exaggerated for one of two colors. These asymmetries indicate the limits of the flat field correction in the lens and should not be observed in the portion of the image collected by the detector. Axial color alignment can be checked by taking images in both color channels for each step in a through-focus series. The maximum bead intensity for both colors should appear at the same focal position. If it is not, then the lens may not be corrected well enough for chromatic aberrations for your application.

Similar to the stage micrometer slide used with brightfield illumination, the bead slide serves as a test slide for determining how the acquisition parameters affect image quality in the fluorescence microscope. A line scan graph through the bead center is a cross section of the PSF, indicating peak signal as it falls off to noise. Taking exposures of increasing duration, or with increasing excitation intensity, will reveal the relative signal to noise capabilities of the microscope and detector under all parameter settings. For example, opening or closing the pinhole in a laser scanning confocal microscope illustrates the relative changes in light collection and PSF distribution. An excellent exercise for acquainting yourself with a new system is to lower the illumination energy to a minimum, and use the detector parameters to maximize the SNR for two closely spaced beads. If you can discover how to do this using the bead slide, finding a good signal to noise compromise for the live specimen is a much easier task.

The best advice that I can give anyone beginning a series of live cell fluorescence experiments is to have a reference bead slide for evaluating the condition of the microscope before you begin each experiment, especially in a multi-user environment. Taking a few images of the bead slide before getting started can save time and data. If the peak bead intensities and the average noise levels from the background differ markedly from the last time you used the microscope, better to find this out before starting your live cell experiment, than after.

6. Determining the image acquisition parameters

Putting it all together to collect data requires planning and a little experimentation. This final section describes steps to-

wards refining the acquisition parameters for an experiment. Where possible, it is of great advantage to have a duplicate or trial specimen that can be sacrificed to pre-tune the acquisition parameters. Before getting started, check the microscope with a bead slide or micrometer slide as discussed above. The objective lenses should be clean and the light path free of optical noise. If a camera is used, determine the spatial sampling rate (between 3 and 8) before starting the experiment. If using a scanning system, determine how to set scan rate and scan size to match the lens resolution. Leave the gain, offset and any read-out amplifier at a moderate setting, allowing for changes that increase or decrease the sensitivity. These are your initial settings for working out the experimental protocol.

Estimate the duration of the experiment

Estimate the amount of time the experiment will take, beginning with specimen collection. This is especially important when reserving time on a multi-user system. Allow time for chamber or perfusion set-up, equilibration in liquid medium, and the actual imaging time. If imaging multiple specimens, decide whether they should be taken from the growth environment together, or should the set-up time be staggered such that each specimen is imaged at the same point in the experimental protocol.

Determine how much of the specimen area to image

Select enough specimen area to provide context for the feature of interest and to account for some specimen drift over the course of the experiment. Select the best magnification for projecting the image to the detector and use either the field diaphragm iris (for brightfield) or the fluorescence illuminator iris (for widefield) to illuminate only that area for imaging. In software, direct the detector to read out only the portion of the frame that you need, reducing image read-out or scan time.

Determine the time interval between image acquisitions

If the experiment requires time-lapsed exposures or taking an axial series of images for 3D reconstruction, you need to know the maximum interval between acquisitions to avoid compromising the data analysis. For example, tracking a mobile feature in the cytoplasm requires an acquisition rate that captures small stops and starts during movement. Creating a 3D image of that feature requires even more images per time interval to ensure that the cellular motion does not impact the feature morphology when the images are combined for the 3D view. At this stage, ignore the effects of over-irradiation. The goal is to clearly demonstrate that the data collection rate will allow you to make the observations you need for publication.

Estimate the maximum exposure time

Creating images with a high SNR over a broad range of intensities is generally easier if the exposure time can be lengthened. Further, some evidence suggests that longer, less intense exposures are less phototoxic to live cells than short, bright exposures, even when the integrated light intensity is equivalent (see Dixit and Cyr, 2003). Therefore, it is advantageous to determine the longest possible exposure time for a specimen that will not be affected by specimen motion or other experimental factors. Again, ignore the effects of over-irradiation at this stage. For some experiments, the maximum exposure time will equal the maximum acquisition interval.

Determine the minimum required illumination intensity

Achieving the experimental goal requires that the SNR be high enough to recognize the feature of interest over the entire duration of the experiment. If the experiment only requires a single time point, and specimen recovery is not an issue, increase the illumination intensity until a maximum SNR and dynamic range are reached for the detector. If multiple time points are required, the excitation intensity must be carefully chosen to maintain a usable SNR over the duration of the experiment, taking the previously determined acquisition time and interval into consideration. If a duplicate specimen is available, a time series of consecutive frames can be taken to estimate the bleaching rate for the illumination setting.

For brightfield imaging, the effects of irradiation level are not always immediately observed, like the photobleaching seen with fluorescence imaging. Find a minimum illumination level for creating an acceptable dynamic range with the detector controls. If plastids stop moving or the cytoplasmic streaming changes in velocity, lower the intensity and consider using a color filter in the illumination path. For live cell work, I recommend a green (540 nm) color filter to reduce activation of blue, red and far red light receptors and to minimize chromatic aberrations.

For fluorescence microscopy, photobleaching and exposure time will limit the excitation intensity. Starting with the maximum exposure time and minimum excitation energy, increase the illumination until the feature of interest can be resolved above noise. Now is the time to work with the detector controls. This might include the read-out amplifier, the gain and offset controls, or pinhole size for confocal scanners. The SNR can be estimated using the line scan tools and the dynamic range is illustrated with a histogram. Try to maximize the number of gray levels above the noise floor with the detector controls. Regardless of how the image rescales for presentation on the monitor, it is the number of intensity levels measured between background and peak signal that determines the primary quality of the image data.

Balancing contrast, resolution and speed

What do you do when there is not enough information in the images to confidently identify the feature of interest? In practice, the signal might fade before taking a good picture or the background/autofluorescence may be higher than the signal. Generating contrast in a live plant cell is difficult because of the high background and the light scattering produced when focusing through a thick specimen. Under these circumstances, many experiments simply cannot be performed as planned. The signal or optical path difference for the feature of interest is below the threshold of detection.

There is typically a moment of unease at this point when trying to decide whether the specimen is really useless or if something can be done with the microscope to extract data for the experiment. Let us assume that you have maximized your chances of collecting data by choosing an optimized filter set and eliminating all colored and fluorescent compounds from the specimen imaging and growth medium. Further, the appropriate lens and technique have been selected, keeping in mind that widefield methods collect more signal but confocal (and DIC) methods remove much of the out of focus background. Lastly, you have used a bead or stage micrometer slide to make certain that the microscope is working properly and to assure yourself that you understand how the individual microscope and detector controls alter contrast and resolution.

The next step is to consider compromises in the acquisition parameters. The goal is to find a useful balance of acquisition speed, resolution and contrast. Beginning with acquisition speed, simply lengthening the exposure time directly increases the signal, at some expense to temporal resolution. If the time interval is not limiting for the experiment, and images can be taken in fast enough succession to prevent slurring, averaging frames together increases SNR as a function of 1/square root of the number of images. Temporal averaging techniques are commonly used in confocal imaging; however, the relatively slow acquisition times for laser scanning confocal images, compounded by taking multiple images per time point, often makes this impractical for live cells. If the specimen is bleaching rapidly, lengthening the acquisition interval will allow you to capture more information per image over the allotted experiment time.

Averaging or summing multiple pixels in the same image improves the SNR at the expense of spatial resolution. Improvement in SNR from averaging follows the same square root relationship as temporal binning above, but the pixels often do not represent signal from the same source (i.e. spatial resolution is lost and the image is blurred). Summing a 2×2 or 3×3 block of pixels in a CCD detector prior to read-out, a process termed binning, has the added advantage that the read-out noise counts only once, while the signal is increased as a multiple of the number of pixels used.

Spatial resolution can also be sacrificed for increased light capture by lowering the magnification to the detector. As the number of pixels per specimen area decreases, the light collected per pixel increases as a squared function. Thus, for a small sacrifice of resolution, a large factor is gained for improving the signal level. For live cell imaging, this is probably the best means of increasing signal. When paired with a change in read-out amplifier, pinhole aperture, or gain and offset parameters, a higher specific SNR can usually be achieved with some loss of resolution.

Increasing spatial resolution involves the opposite trade-off. As more pixels are used to sample the feature, the captured light per pixel drops sharply, requiring either a longer exposure time, higher exposure intensity, opening the confocal pinhole, or a sacrifice in detector SNR through use of a higher gain mode. Contrast is rapidly lost and will often erode resolution as the SNR drops.

Good luck with your experiments.

Materials

Simulated point spread functions and other image manipulations were performed in MATLAB (The Mathworks, Natick, MA, USA). Bead images were taken using a Nikon TE200 microscope (Nikon Instruments USA, Melville, NY, USA) and a Princeton Instruments PI1300 CCD camera (Roper Scientific, Trenton, NJ, USA) using Metamorph software (Molecular Devices, Sunnyvale, CA, USA). Images of AtEb1-GFP expressing Arabidopsis (originally gifted from Jaideep Mathur, University of Guelph) were made using a Leica 63· water immersion objective lens (Leica Microsystems USA, Bannockburn, IL, USA) and a Yokogawa CSU-10 spinning disk confocal head (Yokogawa Electric, Tokyo, Japan). An Cascade 512b EM-CCD camera (Roper Scientific, Tuscan, AR, USA), controlled via Metamorph software, was used to acquire 150 ms exposures using a 16-bit read-out amplifier system.

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