Information Panel

In Vivo Imaging of Mammalian Cells: Image Acquisition and Analysis

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INTRODUCTION

Live cell imaging provides a powerful technique for the analysis of molecular dynamics within cells. Advances in imaging technology and probe design have established this approach as an important tool in modern biology. It is now possible to obtain commercial turnkey systems for digital imaging using a number of different imaging modalities. Nevertheless, it still requires considerable technical care and expertise to conduct a successful experiment. To perform a successful imaging experiment, it is important to maximize the signal-to-noise ratio (S:N) while minimizing damage to the cells. In this article, we focus on the use of fluorescence microscopy in live cell imaging, although most of the points discussed are relevant to any type of imaging. We describe many of the methods and considerations that are required for performing a successful imaging experiment in living cells. However, we do not provide a single recipe for success: The approach is much too empirical and depends on careful observation of the particular cells under investigation.

RELATED INFORMATION

Techniques for the generation and maintenance of fluorescently labeled cells and their use in live cell imaging experiments are described in **In Vivo Imaging of Mammalian Cells: Cell Engineering and Viability** (Swedlow et al. 2009).

DESIGN OF THE ACQUISITION SYSTEM: MAXIMIZING SIGNAL TO NOISE

Live cell imaging usually requires consideration of the relative requirements for resolution and the need to maximize the S:N in the recorded data. First, we discuss the sources of noise and how they may be minimized. Then, we describe the various elements in the optical path, with suggestions for how they may be optimally configured to minimize noise and thereby maximize signal.

Sources of Noise

In imaging, there are four major sources of noise: detector noise, illumination noise, poisson or shot noise, and stray light, each considered in detail below. In general, reducing systematic sources of noise by carefully choosing detectors and optimizing illumination systems can improve the S:N.

Detector Noise

Every photon detector introduces noise into every measurement it makes. The exact details of detector noise are specific to each type of detector. In scanning microscopes (including laser scanning confocal microscopes [LSCMs] and multiphoton microscopes [MPMs]), the photomultiplier tube can produce spurious electrons within the signal amplification system (Art 1995). In widefield microscopes (WFMs) and spinning-disk confocal microscopes (SDCMs), the charge-coupled device (CCD) has a background dark current associated with each pixel. Cooling the CCD and other improvements in CCD design have reduced the contribution of dark current to very low

Adapted from *Live Cell Imaging* (eds. Goldman and Spector). CSHL Press, Cold Spring Harbor, NY, USA, 2004.
Cite as: Cold Spring Harb Protoc; 2009; doi:10.1101/pdb.ip70

levels (Inoué and Spring 1997). However, reading each pixel from the CCD and converting the analog signal to a digital value contribute an additional noise component—the major noise component in the current generation of cooled cameras. This "read noise" is available on the data sheet for any camera (also usually available online, e.g., http://www.roperscientific.com/ and http://www.hamamatsu.com). For live cell imaging using fluorescence, we usually use cooled CCDs with read noise values below 6 e^- RMS.

Illumination Noise

Any digital imaging experiment assumes that the illumination is constant across the sample field and between different images. In most imaging systems, the optical properties across the sample field are usually fairly constant (spatial invariance). However, in scanning microscopes, there may be differences in the illumination dose delivered between different pixels (Zucker and Price 2001; Swedlow et al. 2002). In WFMs, the illumination field can be made fairly uniform by placing an optical fiber between the light source and the microscope illumination path (Kam et al.1993), and any remaining illumination gradient can be corrected computationally by using a flat field correction based on a uniform fluorescent sample (Chen et al. 1995). Nonetheless, an optical fiber does not correct any temporal variations in lamp output. Fluctuations of up to 10% from the mean are common, but they can be corrected using stabilized power supplies and/or by measuring power deviations and correcting them (Chen et al. 1995).

Poisson or Shot Noise

Photon measurement is inherently a statistical process, with an uncertainty associated with signal detection (Art 1995). For a measurement of N photons, the uncertainty is $(N)^{1/2}$, so the S:N is given by $(N)^{1/2}/N$, or $(N)^{1/2}$. Maximizing N leads to an improvement in the S:N. A simple way to improve the S:N then is to collect more signal, usually by taking longer exposures or by increasing the amount of excitation light. Unfortunately, this often is not possible, since these measures usually increase cell photo damage. CCDs can be used to circumvent this problem using a technique known as "binning." Normally, the pixels in a CCD camera are read by transferring a row of pixels into a read register. Each pixel in the read register is then individually read (Abramowitz et al. 2000). In a binned image, each signal from a square group of pixels (2 × 2, 3 × 3, etc.) on the CCD is read out together and assigned to a single pixel (Abramowitz and Davidson 2000). This is achieved by transferring multiple rows into the read register (two rows in 2 × 2 binning, three rows in 3 × 3 binning, etc.) and then reading groups of 2, 3, etc. pixels together. For 2 × 2 binning, there is a twofold loss in resolution, a fourfold increase in signal, and a twofold improvement in the S:N. This improvement can be significant, especially in signal-limited applications. A key point in binning is that the addition of read noise occurs after the binning process, so even though 2 × 2, 3 × 3, etc. pixels are being combined, there is only one read event. If a 2 × 2 box of pixels were computationally averaged after data collection, the result would be inferior to binning because of the noise contribution to each of the four pixels. In summary, binning may sacrifice some spatial resolution, but it provides a significant increase in the S:N, especially when performing experiments on photosensitive cells that require low light levels and short exposures.

Stray Light or Spurious Photons

Recent interest has developed in very high-resolution, high-sensitivity fluorescence imaging in which single fluorescent molecules are detected and tracked over time (Vale et al.1996). This technique requires sensitive detection systems, usually a CCD camera fitted with an intensifier (Inoué and Spring 1997) or more recently, an electron multiplying CCD camera. In fact, the key to making these approaches work has been reducing out-of-focus light using evanescent wave fluorescence excitation and reducing any sources of stray light in the microscope, including those caused by dirty optical elements (Funatsu et al.1997). An important lesson using this technique for all signal-limited imaging is the importance of removing sources of noise in the optical path.

Microscope Optics

Another aspect of optimizing the S:N is the collection and transfer of photons by the microscope optics. Any of the elements in the optical path can degrade the S:N. It is therefore critical to consider the following elements in a microscope designed for live cell imaging.

Objective Lens Numerical Aperture

For fluorescence imaging, it is common to use objective lenses with very high apertures to maximize the amount of light that is collected from the sample. The aperture width is given by the numerical aperture (NA) and is written on the side of all objective lenses. The equation $NA = \eta\sin(\alpha/2)$ defines the NA in terms of η, the refractive index of the medium in front of the lens, and α, the acceptance angle of the lens aperture (for further details, see Inoué and Spring 1997). The resolution of an image recorded with a given objective lens is a function of the NA (Inoué and Spring 1997; Wallace et al. 2001). For fluorescence imaging, however, the light gathering power, and therefore the signal recorded by an objective lens, is proportional to NA^4, so a small increase in NA can yield a significant improvement in signal. It is for this reason that live cell fluorescence imaging often requires high NA lenses and high-refractive index immersion media (e.g., oil, $\eta = 1.51$ or water, $\eta = 1.33$).

Objective Lens Magnification

The brightness of the signal is inversely proportional to the square of the magnification, so it is sometimes worthwhile to consider using a lower magnification objective when imaging dim samples. For example, if the S:N were limiting in a sample imaged with a 100X 1.4NA lens, changing to a 60X 1.4NA, 63X 1.4NA, or even a 40X 1.3NA objective lens would likely provide a significant improvement in the S:N. The loss of resolution will be small compared with the improvement in the S:N. It is always worthwhile to consider magnification and binning together to assess the final magnification necessary for a given experiment.

Objective Lens Correction

Objective lens manufacturers offer a number of different types of lenses. In general, each lens is designed for a limited series of applications; it is advisable to try matching the design principles to the correct application. For example, Olympus, Inc. manufactured a 100X plan-apochromat (PlanApo) 1.4NA objective, a good high-resolution lens. The specification for this lens, however, includes a large amount of chromatic correction so that it can be used for transmitted light techniques, including differential interference contrast (DIC). The result is that light throughput is sacrificed for chromatic correction. This lens has not worked well for us for live cell imaging—its resolution is exceptional, but it does not perform well in signal-limited applications. In contrast, Olympus also made a 100X UPlanApo 1.35NA lens that is not as well corrected (we have detected a small amount of axial chromatic aberration in this lens), but it has significantly higher light throughput, even with a lower NA, because of fewer correcting elements within the lens. Because we rarely attempt high-resolution colocalization in live cell experiments, we sacrifice a small amount of color correction for a significantly improved S:N. Finally, some objective lenses include specific optical elements (such as phase retardation plates for phase contrast optics) that significantly reduce light throughput. This reduction is rarely a problem in phase contrast, but it can significantly reduce the signal in fluorescence. For this reason, we rarely use phase objectives for live cell imaging.

Fluorescence Filter Sets

For fluorescence imaging, interference filters and chromatic mirrors are used to select appropriate wavelengths of light for the chosen fluorophores (Flynn et al. 1998). Because successful live cell imaging depends on the S:N, the choice of filters and chromatic mirrors is absolutely critical for a successful experiment. It is now common to use band-pass filters (Abramowitz and Davidson 1998), rather than simple long-pass filters, to select the fluorophore emission. Band-pass filters are available from Chroma Technology, Inc. and Omega Optical, Inc. for almost any fluorophore or combination of fluorophores. Where possible, choose these for the specific fluorophore(s) being used. For example, using an FITC (fluorescein isothiocyanate) filter set designed for use in a multifluorophore experiment for imaging a living cell labeled with only green fluorescent protein (GFP) will needlessly sacrifice some GFP fluorescence that would help improve the S:N. This is because the filter set is more restrictive than the experiment warrants. Simply choosing the correct filter set can make a significant difference in signal detection.

Aberrations that Reduce the S:N

Any defects in the optical path will affect the S:N in the final image (Inoué and Spring 1997). Spherical aberration is the most common aberration in imaging of living cells with high NA lenses.

This arises from a mismatch between the cells' medium (usually an aqueous medium, $\nu = 1.33$) and the refractive index of the immersion medium. Spherical aberration manifests itself as a misfocusing of the signal, such that objects are significantly elongated along the optical axis (Wallace et al. 2001). Since the signal is spread over a larger volume, the S:N is reduced. This problem can be largely eliminated by using a water immersion lens. Alternatively, the refractive index of the immersion medium or the thickness of the coverslip can be adjusted (Hiraoka et al. 1990). The refractive index changes with temperature, so the optimal combination of immersion medium and coverslip will differ between room temperature and 37°C.

IMAGE ACQUISITION: KEEPING THE CELLS ALIVE AND GETTING THE DATA

Once the cells are appropriately engineered and assembled into an environmental chamber, and the microscope is appropriately configured, one can record image data from the living cells. Deciding on the correct imaging parameters will determine the success of the experiment.

Two-Dimensional Versus Three-Dimensional Imaging

All cells are three-dimensional. Imaging with high NA lenses produces images with a relatively narrow depth of field (~700 nm for a 1.4NA lens), so any single image may only sample a small section of the cell. For some samples (e.g., the leading edge of a migrating cell), imaging at a single focal plane ("optical section") may adequately cover the sample. In other cases (e.g., in the cell nucleus), the object is much thicker and only a portion of an object will be imaged in a given focal plane. Moreover, post-processing methods such as deconvolution usually require sampling of out-of-focus information to generate an accurate reconstruction of the object (Swedlow et al. 1997; McNally et al. 1999; Wallace et al. 2001). These methods can significantly improve contrast, even in images from live cells with a poor S:N (Swedlow et al. 1993). However, recording a series of optical sections subjects cells to significantly more light at each time point. In addition, recording extra images takes time and may significantly slow data collection. This may mean that time points cannot be taken rapidly enough or even that cellular components move during optical sectioning.

Photodamage

During the imaging experiment it is critical to keep light exposure as low as possible. In general, this means reducing the illumination dose using neutral density filters, simply reducing the power of the illumination source, or keeping the time of exposure to fluorescence excitation light as short as possible using shutters or other devices.

Cells are intrinsically photosensitive and adding fluorophores only exacerbates this sensitivity. On excitation of a fluorophore by the absorption of a photon (Flynn et al. 2003), the fluorophore remains in an excited state ("S_1"), usually for a few nanoseconds. "Good" fluorophores will decay back to the ground state by releasing a photon—the fluorescence emission. An S_1 fluorophore, however, can convert to an alternative triplet state ("T_1") that is highly reactive and can cause the formation of free radicals, especially O_2^-. These reactive free radicals can chemically modify cellular substituents, thus damaging the cell. The generation of free radicals and subsequent "photodamage" always occurs during fluorescence excitation and can only be limited, not prevented. Cells have intrinsic enzymatic mechanisms for converting free radicals to less harmful compounds; so as long as these systems are not saturated, cells tolerate fluorescence excitation. In practice, this means that low levels of excitation must always be used during live cell imaging experiments.

Choosing the exact light attenuation and exposure time is almost always an empirical art. The best strategy for a new experiment for which the correct parameters are unknown is to attenuate the light as much as possible (e.g., use a neutral density [ND] filter with an optical density of 1.0) and to use short exposure times (<100 msec) such that the structures are visible in the acquired image, but only barely so. If the cells tolerate this light level through a time-lapse experiment, then the light can be increased in subsequent experiments until a workable compromise is achieved between the S:N and cell viability. It is important to note that there is often a nonlinear relationship between the amount of total light exposure a cell can tolerate and the length of individual exposure times. In our experience, we have found that cells are healthiest when exposed to very brief pulses of light, since extended exposures (>0.5 sec) are often lethal to the cell. In one cell line, for example, attenuating the fluorescence excitation with an ND 1.0 filter and using 100-msec exposures was well tolerated as

long as there was ~0.5 sec between individual exposures. Increasing the rate of exposure to 7/sec, using otherwise identical imaging parameters, caused vesiculation and blebbing. Although the cause of this behavior is unknown, we assume that, after each exposure to fluorescence excitation, cellular antioxidant enzymes must reduce the level of free radicals, and this process takes time. Rapid or long exposures exceeded this limit and thus produced a significant, and in this case unacceptable, dose of free radicals inside the cell.

Variability in Behavior Between Different Cells

Many live cell experiments are performed on one or just a few cells. It is critical to note that cells in culture can be quite heterogeneous and present a wide variety of phenotypes. This heterogeneity can be the result of cells in different positions in the cell cycle or possibly intrinsic differences among cells. A study of transcription activation in a serum-starvation assay highlighted the wide variety of responses among individual cells, even though large differences between control and treatments were scored in a population assay such as an immunoblot (Levsky et al. 2002). For this reason, it is often necessary to record data from many individual cells to gain a statistically significant sampling of cellular behavior and dynamics. Collecting such large data sets can be laborious. In our laboratory, we use a DeltaVision restoration microscope (Applied Precision, LLC) fitted with a three-dimensional motorized stage to record time-lapse images of multiple cells in a single data collection run. This approach has provided enough data to perform statistically valid quantitative analyses of intranuclear dynamics in vivo (Platani et al. 2002). In total, we collected ~350 time-lapse images of GFP-labeled cells under a variety of conditions. Note that this approach generates large amounts of data that challenge standard storage and analysis methods. As a result, we developed informatics tools designed to store and analyze these vast assemblies of image data (Swedlow et al. 2003).

Choice of Acquisition System

A wide variety of digital imaging systems are now available for live cell imaging. The type of system used depends largely on the type of sample and the kind of experiment to be performed. Most systems will behave as reasonably linear signal detection systems, so they can be used for quantitative imaging. WFMs tend to have relatively low intrinsic noise levels and work well with weakly fluorescent samples (Swedlow et al. 2002). For thick samples (i.e., ≥30 µm), however, the amount of out-of-focus light recorded in each focal plane dominates any signal, and better performance is achieved with an LSCM (Inoué 1995; Parry-Hill et al. 2003). An alternative approach is to use a SDCM, where an array of illumination beams rapidly scans the sample and produces a confocal image (Maddox et al. 2003). For very thick samples (i.e., ≥100 µm), the absorption and scatter from the samples become limiting and multiphoton microscopes are the most appropriate system (Helmchen and Denk 2002; Piston and Davidson 2003). The different illumination patterns in these systems are illustrated in Figure 1. In general, it is necessary to try a few of these systems to ascertain the correct approach for a given sample. With the proliferation of these systems in the last few years, it is often possible to visit a nearby laboratory to test whether a specific system works well for a given sample.

RATE OF ACQUISITION

The biological process under study determines how quickly data must be acquired. Ca^{2+} sparks occur over milliseconds, vesicle movement over seconds, mitosis over minutes, and apoptosis over hours. Sampling these different timescales appropriately will require different hardware and software. Before purchasing an imaging system, it is critical to match experimental requirements to the capabilities of the system and to demonstrate that the system can collect meaningful data on an appropriate timescale.

EVALUATING THE RESULTS

Establishing Criteria for Cell Viability

In any live cell imaging experiment, the imaging conditions must be minimally perturbing to the cell and must not significantly affect the experimental result. Most critically, criteria for the health of the cells under study must be developed so that the success of the experiment can be evaluated: not only

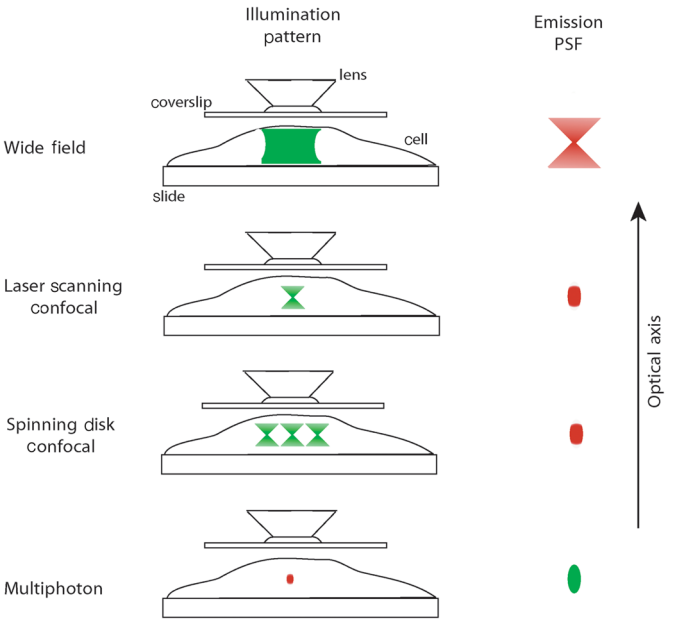

FIGURE 1. Illumination and detection in scanning microscopy and the WFM. The WFM, LSCM, SDCM, and MPM each use different illumination and detection strategies to form an image. The diagram shows a schematic of an objective lens, coverslip, cell, and slide in a typical layout (objects are not to scale). In the center of the figure, the volume of fluorescence excitation for each method is shown. The *right* side of the figure shows the distribution of fluorescence emission from each point source in the sample, observed with the optical axis running vertically on the page. Note that for clarity, the *right* side of the figure has been magnified. The WFM illuminates throughout the field as well as *above* and *below* the focal plane. Each point source is spread into a shape resembling a double-inverted cone known as the point-spread function (PSF) (Inoué and Spring 1997). Only the *central* part of this shape is in focus; the rest is in blurred out-of-focus light. In a real sample filled with point sources, blurred light degrades the image. In contrast, the LSCM, SDCM, and MPM scan the image with a focused laser (Inoué 1995; Helmchen and Denk 2002; Parry-Hill et al. 2003; Piston and Davidson 2003). The pattern of excitation is a PSF, but a pinhole in the optical path in the LSCM and SDCM prevents any out-of-focus fluorescence from hitting the photomultiplier tube detector; therefore, only in-focus light is recorded. The LSCM has a single pinhole and a single focused laser spot that is scanned across the specimen. In the SDCM, an array of holes fitted with microlenses are placed on a spinning disk such that the holes rapidly sweep over the specimen and create an image recorded with an area camera (Maddox et al. 2003). In the MPM, the place at which photon flux is high enough to excite fluorophores with more than one photon is at the in-focus position of the PSF. Thus, fluorophore excitation only occurs in focus. Because all fluorescence emanates from in-focus fluorophores, no pinhole is needed and the emitted fluorescence generates an in-focus image. (Adapted, with permission, from Andrews et al. 2002.)

on whether a given result was achieved, but also on whether the imaging process itself did not damage the cells. The exact criteria used will vary depending on the experiment. Most obviously, the process under study should occur to the same degree and with the same kinetics as that observed in fixed cells. However, it is also important that the conditions used for the experiment (medium, buffering strategy, and chamber) do not substantially change growth rates or mitotic or apoptotic indices. The effects of each of these components should be separately assessed. For example, cells can be grown in the medium used for the imaging experiment in a standard incubator and assessed for viability, mitotic index, etc. In addition, cells can be installed in the environmental chamber for a number of hours and then similarly assessed in the absence of imaging. This strategy isolates the various contributions to cell health and identifies any that should be modified.

During an imaging experiment, cells are exposed to high doses of illumination and, in fluorescence, the generation of reactive species (see above). It is therefore wise to assume that at least some damage has occurred during the experiment and to assess whether it is significant enough to affect basic cellular processes. To do this, after the completion of the imaging experiment, leave the cells in the chamber on the microscope stage and examine them over a number of hours to determine whether they initiate apoptosis or enter and complete mitosis. This is most easily achieved by taking time-lapse images at widely spaced time intervals (10-20 min) over a number of hours. These criteria depend on the appropriate pathways present in the cells used; some mammalian cells have defunct

cell cycle checkpoints, so one should consider the appropriateness of mitotic and apoptotic progression as cell health criteria in a given cell type.

Finally, after an imaging experiment, a very easy assay of cell health is to compare the cells that were imaged with a neighboring field that was not imaged. Do these two populations look comparable by phase or DIC? In the case of fluorescence imaging, is the final localization the same in both populations? If the cells are followed for many hours after the experiment, do the imaged cells behave similarly to the nonimaged ones? These comparisons often reveal whether significant damage has occurred to the cell(s) under study.

Examining Cells in Long-Term Time Lapse

It is often the case that live cell experiments, especially in fluorescence, are performed over relatively short time periods, that is, 30-60 min. Although these studies will reveal exciting results, many of the cells' responses to damage, especially apoptosis, require many hours to develop a visible phenotype. Therefore, to ensure that cells are truly healthy, it is often advisable to image cells to perform an experiment and then simply follow the cells over the next 24 h, preferably by phase or DIC (see Fig. 1). This time-lapse analysis can have relatively crude temporal resolution (e.g., one image every 15 min), but it will reveal whether the cells that have been imaged are reacting to the fluorescence experiment over the long term.

Monitoring Events in Fixed Cell Time Point Assays

Live cell-imaging experiments can be extraordinarily powerful, but it is often very helpful to complement them, where possible, with fixed cell assays to help validate the live cell experiment. This may seem counterintuitive—live cell imaging assays are often held as a "gold standard" for cell and molecular dynamics. However, because of their technical difficulty and the risk of damaging cells during the experiment, demonstrating at least crudely similar kinetics and events in time points from a fixed cell assay can help validate the live cell assay. In some cases, this is simply not possible, either because the kinetics of an event are too fast or because the nature of an experiment (e.g., fluorescent speckle microscopy as a measurement of cytoskeletal dynamics [Waterman-Storer and Danuser 2002]) cannot be meaningfully performed within the fixed cell. Moreover, the point of the live cell experiment is to reveal events or properties that are not observed or easily interpreted in fixed cells. Nonetheless, it is worthwhile to consider the use of this approach as a method of confirming events seen in longer time-lapse experiments to confirm the absence of effects of extended illumination.

ACKNOWLEDGMENTS

We thank members of the Swedlow laboratory for helpful discussions. We are especially grateful to the academic and commercial faculty of the Analytical and Quantitative Light Microscopy and Optical Microscopy in the Biological Sciences Courses (Marine Biological Laboratory) and the In Situ Hybridization, Immunocytochemistry, and Live Cell Imaging Course (Cold Spring Harbor Laboratory) for helpful and stimulating discussions. Work in our laboratory is supported by grants from the Wellcome Trust and Cancer Research, UK. J.R.S is a Wellcome Trust Senior Research Fellow.

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