Building a Live Cell Microscope: What You Need and How to Do It

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ABSTRACT

In this post-genomic era, the predominant focus of biomedical research has moved towards elucidating the functionality of molecules at the cellular and subcellular level in integrated living systems. As such, biologic imaging has moved beyond the collection of static "snapshots" of the cellular state to the real-time visualization of cellular and molecular behavior in three-dimensional (3D) space. Live-cell imaging techniques can be used to assess protein abundance, as well as determine the functional role(s) and interactions of multiple unique molecules concurrently within the cellular environment, and determine the effects of these molecules on cell development, organization, and fate over extended periods of time. Such studies require advanced systems that allow multiparametric analysis of cells while maintaining their functional viability. This unit will focus on the design and implementation of modern live-cell imaging systems. We will examine the principle problems facing live-cell microscopists including: focus drift, cell viability, photo-toxicity, and data acquisition, and suggest appropriate solutions to these issues. Our goal in this unit is to provide individual scientists with the information required to implement live-cell imaging solutions within their own laboratories. *Curr. Protoc. Cytom.* 65:2.21.1-2.21.10. © 2013 by John Wiley & Sons, Inc.

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I. DEFINING THE SYSTEM

In designing and assembling a live-cell imaging system within a particular laboratory environment, it is important to consider integration of the microscope as a whole, such that it is appropriate to the tasks at hand. For example, if time-lapse fluorescence microscopy is the primary function of the instrument, the key factors that need to be considered include: (1) the ability to maintain the cells under appropriate environmental conditions (i.e., 37°C and physiologic pH for mammalian cells); (2) the resolution required to answer the question being asked; (3) the required speed and frequency of image collection; and (4) the need to collect images in multiple fields and/or in multiple image planes. Within this unit, we will discuss each of the fundamental components of the microscope in turn: illumination, objectives, and stage control, as well as the software that integrates the system. Camera choices for widefield and confocal imaging have been covered extensively in an earlier unit and will not be discussed here. We have tried to be entirely objective when considering components, and care has been taken not to endorse any particular product. The reality is that, beyond fundamental considerations, there is enormous variety in the possible approaches to live-cell imaging. The following discussion defines some reasonable expectations for specifically designed systems. To restrict the scope of this unit appropriately, we will focus principally on fluorescent methods for live-cell imaging.

A. Phototoxicity

In any live-cell imaging experiment it is essential that cells be maintained within a homeostatic environment where they can be observed over extended periods. The process of imaging should not lead to any damage to the cells, and ideally there should be no loss of fluorescence from the dyes or proteins used to generate signal. Issues of phototoxicity are generally minor when using transmitted light contrast for imaging, assuming cells are only exposed to light during image collection.

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However, in fluorescence imaging, limiting phototoxicity is generally the most significant factor to be considered in experimental design.

Phototoxicity is the process by which light may kill cells. To some degree that may occur due to incident light being absorbed by intracellular components (lysosomes, pigment etc.) and generating heat, which in turn leads to cell death. However, a more significant problem leading to cellular toxicity occurs during the interaction of light with absorptive fluorophores. In this instance, the excited fluorophore fails to transition back to ground state releasing a lower energy photon. Dependent on the fluorophore and the local environment, the dye molecules may instead transition to a triplet state in which the spin of the excited electron is flipped. The triplet state is a relatively long-lived state, and in cells commonly decays to ground state by interacting with molecular oxygen, leading to the generation of singlet oxygen, which is highly reactive and can ultimately induces protein oxidation, cellular dysfunction and potentially cell death. It is therefore absolutely critical that the first conceptual decision point in microscope design be limiting photon delivery to the cells. Cells should only be exposed to light during image collection, and approaches that do not limit photon delivery should be used minimally. Perhaps the most important thing to recognize is that there is no point in delivering more photons to a specimen than can be absorbed by the fluorophores that are present. Fluorophores have specific decay times, and thus precise control of the photon dose to the specimen should be optimized such that phototoxicity is minimized. Oversaturation of fluorophore by continuing to deliver too much light to the specimen is one of the most common mistakes made in live-cell imaging.

B. Confocal or Not?

This unit will focus primarily on microscope design beyond detection systems and optical sectioning using confocal approaches; however, at this point, it is germane to discuss the complexities of using optical sectioning approaches for live-cell imaging. There is no doubt that standard confocal laser scanning microscopes (CLSM) are ubiquitous in any biomedical research environment, and for many applications, such as fluorescence recovery after photobleaching (FRAP), photoactivation, and uncaging, they are essential, as the approach allows facile laser pointing to bleach, or otherwise perturb or effect the local cellular environment. Further, the point scanner will generate a high-quality focused light image of the cell. In experiments with only a limited number of images, the CLSM is a valuable tool but generally its use should be avoided for live cell experiments. In CLSM, a laser is used to excite fluorophores in a point-to-point manner that delivers a high level of light power to a small cell volume (flux density); this leads to issues of fluorochrome saturation, bleaching, and ultimately phototoxicity as described above. In the hands of the experienced live cell microscopist these effects can be minimized commonly by making compromises in image quality (by increasing scan speed, decreasing pixel count, or decreasing Z resolution by opening the sampling pinhole). Essentially, and for reasons described above, the cellular exposure to light must be minimized. Beyond compromises in image quality, approaches such as 3D sampling should be avoided as much as possible, in all experimental strategies.

Another issue when using standard CLSMs for live-cell imaging is the relatively slow scan speed. To generate high-quality images of cells in multiple colors may take several seconds. In live-cell experiments with compromises in pixel count (generally to 512×512) this may be increased to 4 frames/sec. In an attempt to increase scan speed, and also increase suitability for live-cell imaging, companies (Leica and Nikon) have developed "resonant" scanners, which can scan at higher speeds, and because of the decreased dwell time there is generally less phototoxicity. However, this approach still uses a single pinhole and because scanning is rapid, image quality is definitely compromised.

Multi-pinhole-based confocals, however, have a very important niche within live-cell imaging. These devices traditionally relied on spinning disks (Yokagawa spinning disk) with arrays of multiple fixed pinholes to deliver light to the specimen. The returned light from the specimen passes through an imaging pinhole that rejects the out of focus light. The light is then filtered and delivered to an array-based detector, generally an electron multiplying charge coupled device (EMCCD). There are many, mostly apocryphal, rationales for the decreased phototoxicity found experimentally using spinning disk microscopes. However, the most likely reason is that the cameras used as detectors are far more efficient than the photomultiplier tubes (PMT) found in conventional CLSMs [e.g., a backthinned EMCCD

has a quantum efficiency of 96% whereas a photomultiplier tube (PMT) detector may be only 15% to 25% efficient]; thus, exposures are shorter and the illumination intensity can be significantly reduced. There are multiple companies which market spinning disk solutions, generally using the Yokagawa scanhead, including Perkin Elmer, Andor, Solamere, Intelligent Imaging Innovations, and McBain.

There is no doubt that even using the spinning disk confocal microscopes there can still be significant phototoxicity when compared to wide-field imaging. This is due in part to the fact that the pinhole in the Yokagawa scan head is typically matched to and a 1.4 NA objective and cannot be easily changed. The inability to change pinhole size means that high quality images with less *Z* resolution cannot be collected (as is possible by opening up the pinhole using point scanning systems). Another side effect of the fixed pinhole is that that there are compromises in image quality when lower NA objectives are used (e.g., a 20×, 0.75 NA lens). Both of these issues have been solved using instruments that incorporate an array of pinholes and scan across the image using a single scanning mirror. This approach allows the high speed found in the spinning disk solutions but also allows multiple pinhole sizes or slits to be used. Obviously, if the pinhole is not matched to the NA of the objective, there are compromises in *Z* resolution, but generally (in our hands) this is clearly offset by the ability to collect meaningful images from dim specimens with minimal phototoxicity. Systems that use this technology are sold by Prairie Technologies and Visitech.

Importantly, for most experiments the simplest and least phototoxic approach is to use wide-field imaging. This can be combined with *Z*-sectioning and deconvolution. This approach allows the largest selection of fluorophores to be used, from near UV excitation to the near IR (as the illumination source is not limited to specific laser lines) with the greatest collection efficiency.

C. Upright or Inverted Microscopes

The choice of an upright or inverted imaging platform is very specific to the types of specimens being imaged. This unit is focused specifically to live imaging of cells in culture dishes. When model systems are being imaged using confocal or multiphoton, upright stands are commonly essential in order to access the region of interest. However, for cellular imaging, there are only a few situations when an upright microscope is a superior choice, and this is limited essentially, to patchclamping or other physiology experiments where the large imaging condenser typically found in inverted microscopes limits access to cells. The current availability of *Z*-drift control systems on inverted microscopes (discussed below) and their complete absence on upright microscopes means that the inverted stand is the only logical choice for the majority of live-cell imaging systems.

II. BUILDING A LIVE CELL SCOPE: COMPONENTS AND CONSIDERATIONS

The remainder of this unit breaks down the live-cell microscope into individual components to recommend specific choices for each component where possible.

A. Objectives

The four principal microscope manufacturers (i.e., Leica, Nikon, Olympus, and Zeiss) all manufacture outstanding objectives. These components are at the core of the microscope and careful consideration of which objectives to buy is fundamental to building a high-quality live-cell system. As discussed above, the single most important limiting factor in a live-cell experiment is phototoxicity, and in order to minimize phototoxicity, illumination should be limited as much as possible. However, it is important to generate and collect enough photons from the specimen to produce a useful image. The efficiency of photon collection from the specimen is limited ultimately by the numerical aperture (NA) of the objective. Even objectives with the highest useful NA (1.4) collect only ∼30% of the generated light. Light is lost at every lens element within the objective (approximately 2% per surface). As each lens element has two surfaces, and the losses are compounded as light passes through each lens in the objective, the total loss of light can have a serious and detrimental effect on the final image quality. For example, a high quality plan apochromat optic may lose 30% of the signal, essentially meaning that only a fraction of the photons generated in the specimen exit the objective. Lower NA objectives collect even less of the emitted light such that the efficiency of collection and hence image quality will degrade further. As the NA of any objective is dependent on the refractive index (RI) of the transition media, dry (air, RI = 1.0) objectives have an NA that must be less than 1,

and hence the image quality is less than water or oil immersion objectives, which have higher RIs (~1.3).

What is the best compromise for objective choices (particularly given that high NA objectives are extremely expensive relative to lower NA choices)?

While specific, single-use microscopes may be able to work with only one objective (e.g., if the single use is counting nuclei, a 20× optic may suffice, whereas in order to count yeast a 60× or 100× objective is the best choice), most high-end live cell microscopes will have to image a broad variety of samples. We suggest that the 20×, 40×, and 60× optics offer the most flexibility and greatest economy. A 20× objective can be used to survey cell populations, whereas for subcellular imaging a 60× optic is essential. These objectives should be the highest NA available for the type of transition media used (see above) and have no spherical aberration. Generally, because the quality of objectives currently in production is exceptionally high, plan apochromat objectives are not critical, and plan fluors are usually adequate. Because they have fewer lens elements, the plan fluors have greater light transmission and may be more economic.

When live imaging is being done on cells that must be maintained at 37°C, careful consideration must be given as to whether to use dry objectives or objectives with a transition media such as oil or water. Essentially, the objectives with immersion media pull heat from the cell culture chamber, which may cause problems. This may be avoided by using an objective heater; however, this generally results in condensation on the barrel of the objective and a reduction in the useful life of the objective. Furthermore, water immersion objectives are not useful for long-term experiments as the water will evaporate over time. If multiple XY stage positions are being collected, it is difficult to keep a reasonable amount of transition fluid (oil or water) on the lens during imaging. Finally if *Z*-axis series are being collected, the spring-loading of the lens elements in the objective may distort the front element of the chamber (generally the coverslip on which cells are grown), leading to imaging errors. In order to avoid all these issues, we generally use a combination of dry and oil lenses. We use the highest NA dry objectives available (20× and 40×), as well as 40× (1.3 NA) and 60× (1.4 NA) oil immersion objectives.

We do not use 100× objectives for live-cell applications; apart from imaging yeast, these objectives are usually not necessary on this type of system. Most modern microscopes have an intermediate 1.5× magnifying lens in the stand; either as standard or as an optional extra, effectively making a 60× work as a higher power optic. One reason to avoid using 100× objectives is decreased brightness in the specimen. Brightness in fluorescence imaging increases with the 4th power of NA, and decreases with the square of magnification. In order to optimize the light collecting ability of the system, and minimize light exposure to the specimen, it is critically important to keep the NA as high as possible, without unnecessary increases in magnification. As the 60× and 100× oil objectives have the highest practical NA (1.4), there is only a minor advantage to moving from 60× to 100× in image quality and the resultant signal is always less bright using a 100× objective.

It is also important to consider the light transmission at specific wavelengths when choosing objectives. This is particularly true in the ultraviolet (UV) or near infrared (NIR). For example, the UV excited dye, FURA 2, is a widely used, ratiometric calcium indicator with excitation wavelengths at 340 nm and 380 nm. Most 60×, high NA objectives have relatively poor transmission at 340 nm, whereas the 1.3 NA 40× optics (plan fluor or plan apo) have excellent transmission at this wavelength. This example highlights the importance of ensuring not only that the magnification matches your experimental application, but also that the transmission of the objective is compatible with your choice of fluorescent probes.

The final points to consider when choosing objectives include special requirements for: (1) deep-tissue imaging, which may necessitate water immersion optics, or (2) scanning multi-well plates (96 well or above), which limits the choices to dry objectives. Ultimately, the objectives are the most critical components of any microscope and unfortunately they are also the most expensive

B. System Automation

In addition to a good microscope stand and idealized objectives, other basic components required for a live-cell fluorescent microscope (LCFM) include equipment that will: selectively illuminate specimens with specific wavelengths of light and sort and filter the light before it reaches the camera. In general, these components are highly automated.

C. Illumination Devices

Fluorophores have an increasingly wide variety of excitation and emission spectra. In fact, it is extremely hard to predict what the excitation and emission wavelengths of the next great fluorophore will be. Clearly, scientists developing dyes attempt to match wavelengths to pre-existing and commonly fluorophores [e.g., green fluorescent protein (GFP) became instantly successful as it shared is excitation and emission wavelength with the commonly used dye fluorescein, meaning that anyone with a fluorescent microscope could image it]. However, in order to optimize flexibility, the microscope should be designed such that it can meet the current and future needs of users to image different dyes. This is an important point to consider, as the choice of illumination systems has recently broadened considerably to include not only conventional light sources, but also diode-based illumination systems.

Diodes versus arc lamps versus metal halides

Conventional illumination systems used in fluorescent light microscopes were traditionally built around mercury- or xenon-based arc lamps. These lamps provide bright light across the entire visible spectrum. Notably, while xenon lamps are less bright than mercury bulbs, the brightness is more consistent across the spectral range of the source. These sources have been used since the beginning days of epifluorescent microscopy; however, they are currently becoming much less popular and should not be considered practical when building a modern microscope because the bulbs: (1) are expensive; (2) have a limited lifespan (100 to 200 hr for mercury, 300 to 500 for xenon); (3) do not produce a constant brightness of emission over the full life of the bulb; (4) and take skill to align. These sources are generally provided as original equipment by the microscope manufacturer. For the most part, they have been replaced with metal halide lamps, which are housed in separate cases and the light is delivered to the microscope with a liquid light guide. These are the same price to purchase as arc lamps, and the bulbs remain expensive, but they have several downstream advantages. The metal halide bulbs are: (1) very bright; (2) produce more consistent illumination over time; (3) are much more durable (between 1000 and 2000 hr); and (4) are trivial to align. These sources are made by Sutter, Exfo, Route 89, and most of the microscope manufacturers. Because the bulbs do vary in the intensities of light generated, it is important to make sure that if you wish to use UV or near IR imaging they have robust emissions in these wavelengths.

Most recently, there has been a tremendous expansion in the potential use of diode or diode-pumped illumination sources in optical microscopy. This technology uses diodes or diode-pumped fluors to generate light. When designed properly, it is possible to build light sources that produce extremely bright monochromatic light. The technology has several advantages over other lamp-based approaches. Diodes can be switched on or off in the millisecond time frame, whereas lamps need physical shutters, which take up to 50 millisec to open and close. Diodes have a multi-thousand hour lifespan (typically 10 to 20,000 hr). Diodes do not decay in intensity over time and produce minimal heat. The only significant downside to the use of diodes is that there are currently no UV solutions. In order to maximize the speed advantages of the diode technologies, sources with specific monochromatic emissions are currently the best solutions. Sources of this type are marketed by Zeiss, Lumencor, 89 North, and CoolLED.

Filter wheels

Conventional light sources require filter wheels to select specific excitation wavelengths. Most filter wheels have eight to ten filters and rotate to select the appropriate wavelength. It generally takes ~20 to 50 msec to move between each position, (five positions will take a minimum of 100 msec). The speed varies with the manufacturer. Note that the filter diameter (usually 25 mm) may lead to some vignetting in the image field when viewed by eye (this has no effect on collected image). As the filter wheel is normally combined with a shutter to turn the light on and off when the filter wheel is moving, it is one of the slower components of the microscope. Traditionally, these wheels would be combined with neutral density filter wheels to moderate the light flux from the illumination source. Many investigators no longer use a second wheel, but rather adjust exposure to accommodate for relative brightness. Wheels and shutters are made by LUDL, ASI, Prior, and Sutter, as well as by most of microscope manufacturers. However, the wheels made by the microscope companies, while integrated into the hardware control of the microscope, tend to be

considerably slower than the other commercial sources. There are some other faster color switching solutions that use galvanometers (DG4, Sutter) or monochomators (Till photonics); however, these solutions are no faster than diode sources and suffer from the same issues with spectral stability and bulb life as conventional sources.

Diode sources with multiple discrete lines can be turned on and off very rapidly. Hence, there is no need for a filter wheel or shutter, as these light sources can change wavelength very rapidly. However, they are limited in the number of wavelengths to between 6 or 7 lines, and cannot generate UV light (see above). White light diode sources (Lumencor) do not need a shutter, as they can turn on and off rapidly, but still need a filter wheel to select the appropriate wavelength of light. In terms of cost, a light source with shutters and filter wheel will cost about the same as a diode illumination source. Thus, it becomes a pragmatic decision as to whether spectral flexibility is more important than speed and illumination stability.

Filter cubes and the cube changing turret

The filter cubes lie at the heart of the fluorescent microscope. They sort the excitation and emission light and ultimately define the spectral selectivity of the system. If experimental needs are simple (e.g., only GFP is being imaged), it is not necessary to equip the microscope with a filter wheel, as single cubes can be used. A shutter is still essential to turn the illumination off between exposures, but the design can be made much more cost effective. However, the design of the cube turret still affects the efficiency and utility of the microscope considerably. The first limitation in the functionality of the cube turret is the time it takes to change between cubes. Generally, this is quite slow, up to a second/position. There is no doubt that modern microscopes can change cube positions more rapidly, but it is still one of the most rate-limiting components in the microscopes. Because of this, many investigators use a single polychroic cube that does not need to change positions, coupled to an excitation filter wheel with three or four matched filters. Another limiting factor in the cube changing turret is that all the microscope manufacturers currently use a six position turret. As all the manufacturers use one of the positions to hold the analyzer used for differential interference microscopy (DIC; see below), and a second position must hold a mirror or remain empty if the microscope is equipped with a confocal scan head, there are effectively only four cube positions available for filter cubes. Currently, there are not enough positions in a cube turret for an expanded suite of experiments; therefore, multipass cubes are commonly employed. The multipass cubes are optically far more complex and hence slightly less transmissive than single pass cubes. As they are often not spectrally clean (there is bleedthrough between colors), most live cell microscopes will also have multiple single pass cubes in place. To maximize throughput, and to minimize bleedthrough between colors, it is important to design the filters in the excitation wheel and in the cube turret concurrently, and to purchase all the filters from a single manufacturer after direct consultation. Filters and cubes can be purchased directly from Chroma, Semrock, or Omega or from the manufacturer of the core microscope.

Emission filter wheels

The emission filter wheel is essential in certain situations, but can be avoided in many installations. It is absolutely essential when using emission ratio dyes (e.g., Indo 1 or SNARF). It is also required when using multipass cubes in order to limit issues of spectral bleedthrough. Though not required, the DIC analyzer can be placed in the emission filter wheel rather than sacrificing a cube position in the cube turret for DIC microscopy.

D. *Z* Drift Control

One of the most confounding aspects of live-cell imaging has traditionally been drift in the *Z* axis. The goal of live cell technologies is to establish image collection parameters for both bright field and fluorescence imaging, perhaps in multiple *Z* axis planes, and to allow the microscope to collect images for an extended period of time. Assuming that the cells are maintained in an appropriate physiologic environment (see Culture systems below), this would seem to be a reasonable requirement. However, all microscopes, unless appropriately controlled, are subject to both thermal and mechanical focus drift.

Thermal drift is caused by changes in the microscope configuration due to thermal expansion and contraction of components of the imaging system. Thermal drift has two principal sources: changes in the ambient environment in which the imaging is being conducted (e.g., ventilation and

heating, air conditioning, opening and closing of doors); and changes within the microscope including heat from the illumination source and heat from temperature-controlled stages (see Discussions of illumination and heated chambers above and below respectively). Mechanical drift is the slow progressive loss of focus due to slippage in the primary focus mechanism in the microscope. It may be only a micron each hour; however, this will confound extended term live-cell imaging. Until a few years ago, preventing mechanical drift demanded heroic levels of attention by the investigator during long-term experiments. Fortunately, the problems caused by *Z* drift have now been effectively solved by all of the major microscope manufacturers.

Implementation of *Z* drift correction is different for each manufacturer; however, there are commonalities. All of the *Z* drift correction mechanisms rely on long wavelength light (generally a 780 nm diode) being delivered through the objective and reflecting off the bottom of the dish or slide being imaged and then collected and measured by sensors within the microscope. Any change in the *Z* axis will result in a lateral offset of the collected light thereby changing the measured intensity. The microscope then makes a focus correction to bring the collected light back to the prior intensity either by physically changing focus, or by changing the position of an intermediate lens within the microscope. Implementation of these devices has had an immediate and very real impact on the ease in which long-term time-lapse image series can be collected. While relatively costly (between $10,000 and $20,000), incorporation of a functional *Z* drift controller is considered absolutely essential in any live cell system. There are several questions to ask when assessing the available solutions.

- How smooth is the correction (is it continuous or does it happen sporadically or at timed intervals)?
- Does it work with all the required objectives?
- Does it work with both glass dishes and plastic?
- Do I need to have a high refractive index shift (glass/water) for it to work or will it work with mounted slides?
- Does it work with large changes in *XY* across the dish?
- Can I use different offsets for different *XY* positions?

E. *XYZ* Control

While it is possible to use a simple manual *XY* stage for live-cell imaging, this results in only one experiment/dish. Obviously, this makes data collection from long-term experiments very slow if multiple points are needed. Accordingly, it is more or less essential to use a motorized *XY* stage, which can be computer controlled and allows multiple sequential positions to be imaged. The cost of these devices is closely linked to their reproducibility and speed. They are made by all the microscope manufacturers, as well as multiple independent machine shops (e.g., Prior, Ludl, Sutter, and ASI among others). The *XY* reproducibility is the biggest determinant of cost. If a rotary-encoded stage is used, the controller only looks at the position of the stepping motors, which control the *XY* stage. The accuracy of the rotary-encoded stages is in the micron range. If a linear-encoded stage is used (generally a few thousand dollars more expensive), optical sensors examine the position of the stage independent of the stepping motors, and make positional adjustments accordingly. Generally, the quality of rotary-encoded stages is not very high but images can be aligned post hoc using standard processing software. Thus, while linear encoding is desirable, if cost is an issue, then it is reasonable to invest in a rotary rather than linear-encoded product. That said, the decision should be based on the experimental material that will be routinely used in the laboratory.

Z axis control

Z axis control was traditionally achieved using the focus mechanism in the microscope, commonly using a motorized attachment. Modern digital microscope are all motorized in *Z* and are "fly by wire" such that *Z* axis control is easily achieved at no extra cost. However, *Z* axis control using this solution is relatively slow and piezo electric *Z* drives are commonly incorporated into the *XY* stage to increase speed dramatically. These devices are extremely accurate though the range of travel is restricted, generally to 100 microns (adequate for all cell imaging questions), but can transition through their entire range in millisec and can be synchronized with image capture. For fast imaging in 3D (either confocal or deconvolution), these devices should be considered essential. Piezo electric *Z* drives are made by MCL (Mad City Labs) and PI and can be integrated into products from all the commercial vendors.

F. Control of the Cell Environment

A critical element in the collection of reliable live cell data is maintaining the cells in a physiologically healthy state. The aspects of the cellular environment that must be controlled include temperature, pH, and nutrient availability. In the majority of live-cell imaging experiments, immortalized cell lines will survive experiments using HEPES-buffered media without the need for gassing CO_2 into the chamber. However, primary cultured cells are generally far more sensitive and should be kept in at 5% CO_2, in a humidified environment. The environmental parameters are typically controlled by the use of cell chambers that can be mounted on the stage of the microscope, and a dependable perfusion system. Cell chambers come in three principal varieties: open, boxed, and closed systems.

An open environmental control system is most suitable for short-term experiments or those that require a large volume of medium or addition of an agonist. Beyond standard live-cell imaging, if microinjection or patch clamping is needed, the open system is the only compatible option. The open systems are extremely useful for temperature control in experiments lasting up to 60 min; however, this type of system is impossible to keep sterile and without a continuous perfusion apparatus, the medium is subject to evaporation. Thus, such systems are not useful for experiments longer than one hour. Examples of open environmental temperature control systems are the PDMI-2 Open Perfusion Micro-Incubator (Harvard Apparatus) or the Bioptechs Delta T (Bioptechs).

Large boxed systems use a Plexiglas box to enclose the entire microscope stage. Because of the large air mass in these systems, they can be likened to a real tissue culture incubator on a microscope. They have advantages in that they can be rendered thermally stable, with constant CO_2 and limited airflow. They have disadvantages in that they are difficult to work in, cannot be used in conjunction with microinjection, and are very difficult to clean if contaminated. Examples of this type of box come from companies like Solent.

Smaller box systems are essentially small, low mass incubators with removable lids that sit in the microscope stage. These systems have become increasingly popular as they can be used either as an open system (with the lid off) or as a closed system (with the lid on). They allow careful control of temperature and gas exchange. However, because of the small volume of gas within the chamber, it is difficult to maintain a stable gas mix using a gas mixer, and we, therefore, choose to use premixed gas tanks. This solution is practical, less expensive and tanks typically last a year even under heavy use. Systems using this design are marketed by Tokai-Hit and Okolab, among others. The weight of the boxes varies between vendors. If you are using the microscope *Z* control, this is not an issue. However, with piezo *Z* stages (MCL or PI) or galvanometer-driven *Z* stages (Leica), it is important to make sure that the *Z* axis drive has sufficient power to move the chamber in the stage.

Truly closed systems are relatively uncommon. The most widely used examples are made by Bioptechs (the FCS2) and Harvard Apparatus. In each case, the chambers are sealed in the tissue culture hood and then moved to the microscope to maintain sterility. These chambers are excellent for long-term experiments lasting several days. Further, as the volume in the chamber is small (100 μl), expensive reagents may be delivered into the chamber without dilution. However, it is not possible to physically manipulate the cells within the chamber and shear stress issues have been reported when the flow rates are maintained at high levels.

Except in the case of the truly closed systems described above, the use of disposable glass-bottom petri dishes is encouraged. The self-assembled glass-bottom chambers invariably leak into the microscope, leading to various contamination problems. There are multiple vendors of glass-bottom chambers (e.g., MatTek, ibidi). The standard 35-mm dishes will fit into all live-cell chambers and should be used whenever possible. They are generally economical and are far more reliable and easy to use than any other solution.

G. Software

The automated control of the microscope and associated devices, and image acquisition, are increasingly platform dependent. All of the four microscope manufacturers have developed their own software packages (Leica Application Suite, Nikon NIS-Elements, Olympus Soft Imaging Solutions, and Zeiss AxioVision). These software packages control all of their own microscope systems. Beyond this, there are several state of the art packages from independent software companies including Metamorph (Molecular Devices), Image-Pro (Media Cybernetics), and SlideBook (Intelligent Imaging Innovations). Finally, there

are packages available from camera companies (e.g., Photometrics, Andor, and Hamamatsu) that offer some basic imaging capabilities. The software packages provided by the microscope companies are highly capable of performing a large array of microscope control functions and analysis. The third-party software tends to be significantly more expensive than that provided by the microscope companies, though in most cases these packages are exceptionally powerful. The key decision point is whether to use a single software platform for acquisition and post hoc analysis. If you decide to go this route, and to use the microscope vendor's software, it is evident that you must continue to work with a single microscope vendor when you purchase additional systems. If you use a third-party vendor for software, you will be able to keep a single software platform between all the microscopes you have within your laboratory, independent of manufacturer. Importantly, the software used on standard microscope platforms and spinning disk systems is not always the same as that used to control standard point scanning confocals for Zeiss or Olympus microscopes. Leica and Nikon use a single common platform for all their commercial devices. It is common for imaging laboratories to have a single platform for acquisition and then use a different single platform for analysis (this is either because of a legacy purchase, or because off-line packages are generally much less expensive than online packages). When purchasing software, it is also very important to define the annual cost of ownership to maintain the software licenses from year to year, as the capabilities of, and techniques embedded in, the software for both acquisition and analysis change rapidly.

III. TRANSMITTED LIGHT CHOICES

While this unit focuses on a discussion of fluorescent methodologies in live-cell imaging, transmitted light illumination is essential to any live cell system. The least case scenario is that transmitted light is used only to validate the specimen. However, we find it is a vital component of many methods, such as monitoring of cell motility, or transfection or infection efficiency. As live-cell imaging does not allow contrast of cells and tissues using traditional staining methods, other brightfield contrasting methods must be employed. We will discuss these methods only briefly, principally examining how the different methods affect the efficiency of fluorescent light excitation and detection of the emitted fluorescent signal. The two principal methods are phase contrast and differential interference contrast (DIC).

A. Phase Contrast

Developed by Zernike, this method depends on the presence of a phase plate within the objective. As fluorescence imaging uses the objective for transmission of both the exciting light and emitted signal, this phase plate can have a significant impact on the amount of fluorescent signal recovered (up to 30% of the available signal may be lost). For this reason, we avoid this contrast method for any instrument in which we are specifically trying to detect low level fluorescent signal. Phase contrast is useful when trying to image cytoskeletal components in transmitted light, as these cellular features generally provide only minimal contrast using DIC. Importantly, phase contrast does not allow optical sectioning of the cells in the image field. It is less expensive to implement than differential interference contrast (DIC), but generally we feel that DIC is a far better and more flexible solution.

B. Differential Interference Contrast (DIC)

DIC is the preferred method for high-resolution transmitted light imaging in live-cell microscopy. The three main DIC methods are Normarski, Schmidt, and de Sénarmont. DIC depends on polarized light that is effectively split using a Wollaston prism housed within the microscope and made of birefringent material (e.g., calcite). The light then passes through the sample and is recombined by a second Wollaston prism. Lastly, it is passed through a final polarizing filter called the analyzer. The contrast in DIC is dependent on differences in refractive indices for various cellular components, and thus differences in interference as the light passes through cells. Thus, the edges of cells where there is both lipid and cytoplasm, which have dramatically different effects on the light, will provide extremely high contrast information. On the other hand, within the cells, little contrast is seen. This technique also provides exquisite resolution of cellular motion. The Wollaston prism has little effect on light transmission except when performing imaging at the diffraction limit, where the diffraction-limited objects show significant aberration (e.g., in TIRF imaging). Under these conditions, the objective Wollaston prism should be removed. The

polarizing, analyzer filter will decrease fluorescence signal (for both excitation and emission) considerably. Fortunately, in contrast to phase-contrast microscopy in which the phase ring is contained within the objective, it is possible to remove the analyzer when it is not being used; in fact most current microscopes use a turret position for the analyzer such that it must be removed before fluorescence imaging. Thus, an essential component of many live cell systems is the possibility of removing the analyzer during fluorescence detection.

DIC has a further advantage in that it optically sections cells, thus is an essential component of confocal microscopes (point scanning, not spinning disk). However, it does have two significant limitations, the first of which is cost. It generally costs between $5,000 and $10,000 to implement this method within any microscope. The second, and perhaps more important, limitation is that this method cannot be used to look at cells within a plastic culture dish because polarization is lost when light passes through plastic. This is perhaps the prime reason why investigators still choose phase-contrast microscopy, or Hoffman microscopy. Hoffman contrast produces a DIC-like effect; however, like phase, it demands specific inserts within the objective, which severely limit use in fluorescence-based live-cell imaging. However, when imaging cells in physiologic experiments such as patch clamping or microinjection, Hoffman contrast is extremely useful.

SUMMARY

This unit serves as a very brief summary of the mechanics of live-cell imaging. It is important to recognize that individual systems must be designed very carefully for the intended application, and this should only be done by an experienced microscopist; otherwise, extremely costly mistakes may result. Most instrument vendors will provide individualized solutions; however, demonstrations of utility and functionality within the environment and using the relevant cells and fluorophores are essential to assembling a system that will perform successfully over many years.