CHAPTER

10

Introduction to Fluorescence Imaging of Live Cells: An Annotated Checklist

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It is becoming clear that most biological molecules in living cells are in a highly dynamic state, changing their interactions and spatial organizations in response to signals. With recent advances in optics, probe design, and photon detection, there are few approaches as suitable as fluorescence imaging for investigating dynamic events in living cells. However, successful execution of fluorescence imaging relies heavily on proper setup of facilities. The purpose of this article is to provide a comprehensive list of the equipment, with associated notes, for those setting up new imaging facilities.

There are many options in setting up a fluorescence imaging facility, each with its advantages and disadvantages. The decision is usually dictated by a combination of experimental goals and personal preferences. While this article attempts to provide some useful guidelines, it is important not to equip much beyond what is necessary, as excess equipment wastes money, creates confusion, and often becomes obsolete when one finally finds the opportunity to use it.

Microscope Stand

An inverted microscope generally allows more flexibility and workspace for the culture (see later) and manipulation of live cells than an upright microscope. In addition, while inverted stands by major manufacturers give comparable performances, it is important to take into consideration the feasibility, accessibility, and convenience for special third-party accessories, such as the culture chamber, micromanipulator, filter wheels, and confocal optics to be used. Therefore, choice of the stand should not be made until the design of the rest of the system becomes largely clear.

One aspect particularly relevant to living cell imaging is the stability of the microscope stand. Some old microscopes are prone to stage drift, particularly at an elevated temperature. They require either extensive manual input or an autofocusing mechanism for time-lapse imaging. However, it is often difficult to obtain reliable information on the stability through manufacturers or on-site demonstration, and experience of colleagues is often the most reliable source.

Several current microscope designs incorporate useful automatic features, such as lamp shutters (see later) and motorized magnification and stage controls, which may alleviate the need to incorporate third-party components and facilitate automated multimode time-lapse imaging.

Objective Lenses and Contrasting Method

A basic set of objectives consists of 10×, 40× dry, 40× immersion, 100× immersion lenses and possibly a 60 or 63× immersion lens. Dry lenses are used primarily for scanning the samples and do not have to be expensive. However, immersion lenses should have as high a numerical aperture and light transmission efficiency as possible. Because images are typically collected near the center of the field, lenses highly corrected for flat field usually provide no detectable benefit and are more costly and less light efficient than simpler lenses such as Fluar lenses. All lenses for fluorescence imaging should be checked upon delivery for the quality of point-spread function, using fluorescent beads as the sample (see later).

For most applications, phase-contrast optics should suffice for scanning the sample and for collecting paired fluorescence and transmission images. The presence of quarter-wave plate in the phase lens does cause some (~<5%) light loss, although it is usually not serious enough to defeat the use of phase-contrast optics. The alternative is DIC optics, which requires

the repeated insertion and removal of an analyzer in the optical path when one shifts between DIC and fluorescence optics. This, and the higher cost, makes DIC optics less desirable in most cases.

Condenser

Unless the experiment involves high-resolution transmission optics or dark-field optics, a condenser with a long working distance should be used in conjunction with an inverted stand.

Control of Projection Magnification

It is critical to choose an optimal magnification for live cell imaging, balancing between signal intensity (favored by low magnification) and resolution (favored by high magnification). It is particularly important to match the final magnification with the pixel size of the detector (see later). Projection magnification may be controlled conveniently in some microscopes by switching the tube lens or by adding additional lenses (referred to as Optovar for Zeiss microscopes). New stands allow changes of magnification to be controlled automatically in time-lapse imaging.

Epi-illuminator and Fluorescence Filter Sets

Some new epi-illumination systems provide a light trap, which reduces the background stray light, and standard Kohler illumination with both field and aperture diaphragms. The aperture diaphragm may be used to control the lamp intensity as well as the angular span and depth of illumination. The light trap is useful for single molecular imaging, which is typically limited by the background.

There are several commercial sources of high-quality fluorescence filter sets. A handbook on the selection of filter sets may be found at the Web site of Chroma Optics. The main consideration is to balance signal strength (favored by cuton/cutoff filters or wide band-pass filters) against the reduction of signal crossover from probes of different colors (favored by narrow band-pass filters). The latter consideration is particularly important when an intense, long wavelength probe is used in conjunction with a weak, short wavelength probe. In addition to standard filter sets, multiband filters are now readily available that allow simultaneous illumination and/or detection of multiple fluorophores.

Heater Filter or Heat Mirror

When imaging living cells it is critical to remove the infrared component from the light source, as the fluorescence filter set may not be able to block infrared light. Failure to do so may cause not only heat damage to the cell, but also high background with some infrared-sensitive cameras. The filter (e.g., BG38) or heat-reflecting mirror may be placed either in front of the lamp or in the epi-illuminator. Attention should be paid to the UV transmission of these filters if UV excitation is to be used for imaging.

Lamps and Lamp Power Supplies

The system should include both a mercury arc lamp and a 100-W quartz-halogen lamp for epi-illumination, coupled through a selection mirror to the microscope. Contrary to common practice, the most suitable lamp for fluorescence imaging of live cells is often a 100-W quartz-halogen lamp. Unless the experiment involves single molecule or speckle imaging, quartz-halogen lamps are much more cost effective and are sufficiently intense for imaging most cellular structures while minimizing radiation damage. They also allow easy adjustment of the light intensity, using a variable, stabilized DC power supply.

The mercury arc lamp should consist of a well-shielded housing, power supply, and power supply cable to minimize the potentially damaging electromagnetic wave during ignition. Although some power supplies allow adjustment of the light intensity, the range of adjustment is limited. Therefore, it is often necessary to attenuate the light from mercury arc lamps using a set of neutral density filters to avoid radiation damage. Alternatively, the intensity may be controlled using the aperture diaphragm in the epi-illuminator as mentioned earlier.

Shutters

Electronic shutters should be used to control fluorescence excitation. These shutters should be used as much as possible to minimize the duration of illumination and should have the interface for computer control during automated time-lapse recording.

Cameras

Cooled CCD cameras are used for most fluorescent imaging applications. In choosing a camera, important parameters include quantum efficiency, noise level, pixel size and full-well capacity, and scanning frequency. In order to minimize the excitation light for imaging live cells, the camera should be as "sensitive" as possible, which generally means a high quantum efficiency, low noise, large pixel size, and slow scan rate. The sensitivity requirement must therefore be balanced against the required resolution (favored by a larger number of small pixels) and imaging rate (favored by a higher scanning frequency).

Slow-scan CCD cameras are generally limited in their frame rate. In addition, unless the sample is very intense, the signal-to-noise ratio is poor under short

exposures. This limits both their use for high-speed imaging and the ease in focusing the images. Focusing is facilitated with cameras using the shutterless, frame-transfer or interline CCDs, which are able to provide a continuous stream of images at video rate in addition to slow-scan digital signals.

Intensified CCD cameras are generally more suitable for high-speed imaging, although usually with a compromised quantum efficiency. Of particular interest are cameras that use CCD chips with the new electron amplification technology (e.g., photometric Cascade and Andor Ixon cameras), which allow both long-exposure and high-speed imaging at a high quantum efficiency.

Optical Coupling of Cameras

Coupling with the detector should be achieved with as few lens elements as possible. It is recommended to have several couplers with different magnification factors. In conjunction with different objective lenses and projection magnifications mentioned earlier, they allow a wide range of magnifications for both light-limiting and high-resolution applications. The Nyquist resolution criterion should be considered when choosing the magnification: each pixel should correspond to no more than half the required resolution limit on the sample. Due to the diffraction limit of the microscope, this distance needs not be smaller than 50 nm for most applications. The actual area imaged onto each pixel may be determined easily by taking an image of a scale standard (see later).

Vibration Isolation Table

A full-fledged vibration isolation table is necessary in adverse environment, e.g., in areas of heavy traffic or in high-rise buildings, or for demanding experiments of micromanipulations or single molecular imaging. Simple isolation measures may suffice otherwise. These include inner tires under the table or rubber isolation pads (Edmond Scientific) or tennis balls under a slab tabletop.

Motorized Stage and Focusing Control

A motorized XY stage is optional. It allows one to monitor multiple cells in separate regions and may increase the output greatly in time-lapse experiments. A motorized focusing control is required for optical sectioning, three-dimensional imaging, and automatic focusing. However, many simple imaging experiments may be better served without the complications of motorized stage controls.

Computer Hardware and Software

A high-end personal computer is required not only for image acquisition, but also for device control and data analysis. The system should have a high-capacity hard disk, a recordable CD/DVD drive for archiving and porting data, and double monitors to accommodate all the images and control windows. Before making a decision on the software package, it is advisable to prepare a list of application requirements, as many features seen in software demonstrations are visually dazzling but practically useless. The efficiency of a package should be judged by counting the number of mouse clicks or key strokes for setting up and triggering the most frequently used functions.

As a minimum for live cell imaging, the program should be able to control the camera, shutters, and motorized devices, to perform time-lapse recording, and to allow changes of recording parameters without stopping the recording. In addition, the user should be able to review dynamic processes as movies even during the recording. The program should also be able to perform automatic contrast enhancement (without losing the original intensity values) and to save images in a nonlossy file format with automatically generated or manually entered file names.

Microscope Incubators

There are a number of options available through microscope manufacturers and independent companies such as Bioptechs. Discussions of cell culture on microscopes may be found at the Web site of Bioptechs and in McKenna and Wang (1989).

There is no "ideal" culture device for all the applications. The range of possible devices varies from a heated stage, a heating collar for the objective lens, a small heated culture chamber, to a large enclosure that fits over the entire microscope. A heated stage is the most convenient but the least functional, as the point of observation is over an open area far away from the heat source. A heating collar for the objective lens applies heat much closer to the sample. However, it works only in conjunction with immersion lenses and has a limited area of heating. It is usually used in conjunction with an additional chamber or enclosure. Large microscope enclosures provide the most stable temperature; however, it must be designed carefully to provide convenient access. There are also a number of perfusion and heated culture chambers for microscopy. These chambers generally provide excellent optical and culture conditions; however, the associated wires and tubing may add to the inconvenience, and the sealed environment may not be compatible with micromanipulation experiments.

It is important to maintain the temperature stability to within a fraction of a degree, as even minor drifts in temperature can cause severe drifts in focusing and

sample positioning. In addition to heating, it is important to maintain the pH and osmolarity of the culture medium. While this should not be a problem with sealed chambers or perfusion chambers, open chambers should be used in conjunction with either a CO_2-independent medium (e.g., L-15) or injection of CO_2 into the incubator. It should be noted that HEPES-buffered media only slow down pH drift and are not suitable for long-term cultures by themselves. Osmolarity may also be a serious problem with open dishes in a heated environment. It may be controlled by replacing the medium periodically or by covering the medium with a layer of mineral oil (Sigma).

Testing Samples

Several testing samples should be prepared for characterizing the imaging system. First, a micrometer scale is essential for all microscopy laboratories for determining the final magnification. Second, a "flat field" sample is prepared by spreading a drop of appropriate fluorophores in 50% glycerol under a coverslip. It is useful for checking the uniformity of epi-fluorescence illumination and for setting the field diaphragm. Also essential is a sample of fluorescent beads. It is prepared by diluting 0.1-μm-diameter fluorescent latex beads (Molecular Probes) by ~10^5 into melt 1% agarose, and mounting a small volume (~20–50 μl) of the suspension on a heated glass slide under a coverslip, before letting the sample cool down. The bead sample is used for checking the combined optical quality of the imaging system. Defects not readily visible with cell samples are often recognized easily when one examines the images of single beads. Defocused beads should appear as radially symmetric disks or concentric rings.

Additional Equipment

Additional equipment may be required for special purposes. Multiwavelength imaging is often achieved with filter wheels at the illumination and/or detection optical path. In addition, devices such as Dual-View and Quad-View from Optical Insights generate composite images at different wavelengths. Spinning disk confocal heads have been used extensively for imaging fine structures in single cells. Its balance between light efficiency and resolution proves particularly suitable for cultured cells. Finally, total internal reflection fluorescence optics is being used extensively for imaging structures near the cell–glass interface, such as focal adhesions. Details of these approaches and devices are beyond the scope of this article; however, their potential use should be considered when designing the imaging system.

Reference

McKenna, N. M., and Wang, Y.-L. (1989). Culturing cells on the microscope stage. *Methods Cell Biol.* **29**, 295–305.