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Using Fluorescence Microscopy to Study Mitosis

Sai K. Balchand, Barbara J. Mann, and Patricia Wadsworth

Department of Biology and Program in Molecular and Cellular Biology, University of Massachusetts Amherst, Amherst, MA 01003

Summary

Fluorescence microscopy is one of the most important approaches in the cell biologist's toolbox for studying the mitotic spindle. In fact, many of the key insights into our understanding of mitosis have been enabled by the visualization of mitotic processes using fluorescence microscopy. Here, we summarize some of the important considerations for imaging mitosis using fluorescence microscopy. Because light can damage live cells, we emphasize the importance of minimizing cellular damage while obtaining informative images.

Keywords

Spinning disc confocal microscopy; Confocal laser scanning microscopy; Mitosis

1. INTRODUCTION

The impact of fluorescence microscopy on cell biology in general, and the field of mitosis in particular, is profound. This major impact has occurred due to the confluence of the development of fluorescent reporter molecules that can be delivered to and expressed in live cells and advances in equipment used for imaging. Over the past few decades, major improvements in detectors, light sources, filters, objective lenses, and the increased availability of turnkey systems for confocal microscopy, have provided powerful tools to the researcher seeking to image the events of mitosis. Researchers have taken advantage of sophisticated new equipment to image cells that have been engineered to express mitotic proteins tagged with genetically encoded fluorescent proteins, FRET sensors and other fluorescent markers. Methods for introducing engineered proteins into cells have been greatly improved, enabling the researcher to control level of expression (1) and to perform near simultaneous imaging of multiple fluorescent proteins in the spindle. Moreover, recent introduction of gene editing protocols for mammalian cells promises to revolutionize imaging of tagged proteins by permitting modification of the endogenous genetic locus with fluorescent tags (2). These new developments will undoubtedly provide unanticipated insights into mitosis.

Obtaining meaningful, quantitative data with the fluorescence light microscope requires a thorough understanding of the many variables that must be controlled during imaging (3).

Perhaps the most important consideration is that the cells must be kept healthy and dividing throughout the acquisition process (4). Unfortunately, light is not inert and will cause damage to the live cells if not used judiciously. Fortunately, many key parameters of the experimental design for successful live cell imaging using fluorescence microscopy have been worked out (5); similarly, protocols for generating cell lines expressing tagged proteins are also available (6,7). Here, we outline methods for fluorescence microscopy of mitosis in mammalian cultured cells, including maintaining cell viability. With minor modifications, similar methods can also be used for other cultured cells from other species (e.g. *Drosophila* S2 cells) and other model systems (e.g. *C. elegans* embryos).

2. MATERIALS

1. Non-CO₂ medium: culture medium without phenol red indicator dye and without bicarbonate buffer and containing 20mM HEPES pH 7.2 and supplemented with Fetal Bovine Serum (FBS).
2. Oxygen scavenging system, e.g. Oxyrase.
3. Sterile glass coverslips, thickness #1.5.
4. Chambers for holding coverslips or glass bottom culture dishes.
5. Thermistor probe.
6. Vacuum grease or mineral oil.
7. Lens cleaner.

3. METHODS

3.1 MAINTENANCE OF MITOTIC CELL HEALTH

It is of utmost importance that cell health is maintained during imaging. In the case of imaging mitotic cells, this can be easily evaluated by comparing the progress through mitosis of the imaged cells with appropriate control cells that were not exposed to fluorescent light. Successful completion of mitosis, with kinetics similar to the control cells, is a good indication that the imaging protocol has not caused cellular damage. Whenever possible, mitotic progression should be evaluated for the imaging conditions used for your experiments.

PROTOCOL 1: Maintaining mitotic progression during imaging

1. Plate the cells in appropriate dishes or on coverslips for use in closed chambers (Note ¹). Coverslips should be flame sterilized or autoclaved prior to use; #1.5

¹Two basic chamber designs are typically used for imaging cells by fluorescence microscopy: glass bottom cell culture dishes and closed chambers that hold a coverslip (4). Of the two, the dish with a coverslip bottom (e.g. MatTek dishes) is the simplest. Cells are plated (at the desired density) in the dishes, which come in various sizes, 1–2 days prior to experimentation. The main drawback of the dishes is evaporation of the medium and the resulting condensation on the lid. Evaporation can be reduced by placing a layer of mineral oil on the top of medium in the dish or by using vacuum grease to seal the lid to the dish bottom. Another drawback is that the dishes are not always perfectly flat or stable depending on the stage holder, and the lack of stability raises the possibility of spills from

glass is the proper thickness for high numerical aperture objective lenses. Cells are typically plated 1–2 days prior to experimentation.

2. Choose an appropriate stage/microscope heater system and pre-warm to the desired temperature (Note ²).
3. Remove the cell culture medium and replace with pre-warmed Non-CO₂ medium that lacks bicarbonate buffer and contains 20mM HEPES, pH 7.2 and supplemented with FBS. Non-CO₂ culture medium lacks phenol red, which is autofluorescent.
4. Add an oxygen scavenging system to retard photobleaching (e.g. Oxyrase). Because Oxyrase contains bacterial membrane fragments, it is added to the medium, and then filtered, using a syringe filter, prior to adding to the chamber or dish containing the cells.
5. Bring the cells to the microscope for imaging.

3.2 FLUORESCENCE MICROSCOPY OF MITOTIC CELLS

Choosing an imaging system—For a number of years, imaging mitotic cells by spinning disc confocal microscopy (SDCM) has been the gold standard in the field, primarily because the rapid acquisition possible with this type of confocal system enables the dynamics of the mitotic spindle to be captured (8). However, laser scanning confocal microscopes (LSCM) can also capture dynamic events, especially instruments equipped with resonant scanning mirrors. LSCMs have additional advantages, including the ability to change pinhole size, to increase magnification using electronic zoom, spectral detectors to enable imaging with a wide range of fluorophores and built-in capacity for photobleaching and photoactivation experiments (3). For a discussion of some of the major differences between these types of confocal systems, and the option of imaging using wide-field fluorescence followed by deconvolution, see Note ³.

the dish onto the microscope stage or objective lens. The advantage of the dishes is primarily the ease of use and the fact that inhibitors, or other reagents, can be added by simply lifting the top of the dish and exchanging the medium. As an alternative to an open dish, we and others use closed chambers. One example is the Rose Chamber (4,9), which consists of two metal plates and a middle spacer that have a central hole. The coverslip is placed on the bottom plate, covered with the spacer, the top plate is added and the entire assembly is screwed together. Medium is added and a top coverslip (circular, 25mm diameter) which acts at the lid. (Alternatively, the top coverslip can be placed beneath the top plate and medium can be added and removed using two syringes and needles inserted into the spacer). All parts of the chamber can be autoclaved if it is important to maintain sterility. We find that cell health is improved in these closed chambers and that the metal, once warmed to 37 °C, retains heat better than a plastic dish, resulting in reduced temperature fluctuations. Additionally, the chambers rarely leak. The chambers can be placed in an incubator, after the experiment, for subsequent observation, and the top lid can be removed for adding inhibitors or fixative. The chambers can be home fabricated in a machine shop; similar chambers are commercially available. Although there is up-front expense, the chambers can be reused for years, eliminating the need for disposable plastic dishes.

²Mammalian cells are typically maintained at 37° C during imaging, although cell division will continue slower at somewhat lower temperatures (30–36 °C). Microscope manufacturers provide a number of options for regulating temperature, ranging from environmental chambers that enclose all or a large portion of the microscope to smaller stage heaters, or air heating devices (4). Environmental chambers that also regulate humidity and gas level, which need to be controlled when imaging for long periods, can also be used. If such an environmental chamber is not available or desired (some users find that the large enclosure impedes access to the microscope), we find that a stage heater is an excellent choice, especially when used along with an objective lens collar that warms the lens so that it does not function as a heat sink. To measure the temperature during imaging, use a Thermister probe with a micro-sensor that can be placed in the culture dish. This will provide a much more accurate measure of the temperature in the dish than the read-out of the stage heater.

³Selecting a Microscope system. Because several different types of fluorescence microscope systems can be used for imaging mitotic cells, understanding their principle features is important to make an informed choice. The first issue is to determine if a conventional wide field or a confocal microscope is required. The principle advantage of confocal microscopy is the capacity to remove fluorescence light coming from focal planes above and below the plane of focus. By removing this light, the ability to detect fine

PROTOCOL 2: Imaging mitosis using Spinning Disc Confocal Microscopy

1. Turn on microscope system and follow the manufacturer's recommendation for warming up the lasers. This protocol assumes familiarity with the basic software commands for the system you are using.
2. Bring a dish of cells to the microscope in pre-warmed non-CO₂ medium and secure the dish to the microscope stage. Because cells are not under ideal conditions, i.e. they are in a non-CO₂ buffered environment, switch to a new dish of cells after about 1 hour of imaging.
3. Select an appropriate objective lens. Using an objective lens with high numerical aperture and corrected for chromatic aberration gives the best possible results. Most SDCM systems have pinholes that are optimized for higher magnification lenses; newer systems have a choice of pinholes, enabling imaging at a range of magnifications.

structure is greatly improved. However, it is important to recognize that confocal imaging per se does not significantly change the resolution limit of the light microscope, assuming all other factors are the same, e.g. objective lens NA. Because the mitotic spindle is a three-dimensional structure, the advantage of using a confocal microscope to remove out of focus fluorescence is significant (3). That being said, a similar improvement can be accomplished by collecting a Z-series of images with a conventional wide field microscope, followed by deconvolution to remove out of focus fluorescence (10). In fact some would argue that wide field microscopy and deconvolution offer important advantages over confocal, including speed of acquisition and the fact that essentially all the light is collected, whereas in confocal, some light is rejected at the pinhole. For additional information the reader is directed to <http://micro.magnet.fsu.edu/primer/>.

A laser scanning confocal microscope (LSCM) is built on a conventional optical microscope stand. To generate an image, laser light, focused by the objective lens, is raster scanned across the sample. Fluorescence light emitted from the sample is collected, also by the objective lens, and passes through a pinhole, which removes fluorescence coming from above and below the plane of focus. The detector is a sensitive photomultiplier tube (PMT). The scanning, collection and display of the resulting image is under computer control. To obtain information from different Z depths of the sample, the microscope has a motorized Z control (accomplished by moving the objective lens or the stage). Thus, the user can generate a Z-stack of images; the thickness of each plane in Z is controlled by the diameter of the pinhole and the number of discrete images taken.

Modern, turnkey LSCM systems are equipped with multiple solid-state or gas lasers, each generating a specific wavelength (or wavelengths) of light. In practice this means that multiple fluorophores can be excited and imaged, either sequentially or in some cases simultaneously. Additionally, many LSCM systems are equipped with spectral detectors that can distinguish fluorophores by their emission spectra. Thus an important advantage of such systems is their use in experiments that require use of several probes or for experiments such as FRET where there is some spectral overlap.

Because the laser light scans the sample, LSCM systems are well suited for both photoactivation and photobleaching experiments. Using software controls, local activation or bleaching can be easily performed by selective scanning of a predetermined region at the appropriate laser wavelength and power. For more information the reader is directed to Chapters 2 and 3 of this volume.

Obtaining the best images with a LSCM system requires understanding of the various parameters that can be controlled during imaging. For example, by controlling the laser scan speed the amount of excitation light illuminating (and collected from) the sample can be varied; slower scanning results in more light collected, but more bleaching. Rapid scan rates typically generate noisier images; to reduce noise, multiple scans can be averaged. The laser power can be regulated independently of scan speed and averaging. Finally, LSCM systems have a zoom feature that allows the user to decrease the area that is scanned, essentially allowing for a range of magnifications from a single objective lens. Generally, for live cell imaging, less light (lower laser power) and faster acquisition (rapid scan speed) is preferred to keep the cells dividing, and typically this must be determined empirically.

The major alternative to a LSCM is a Spinning Disc confocal microscope (SDCM). The major difference between the two types of confocals is that in a SDCM multiple points of laser light scan the sample simultaneously, thus typically reducing the time required to collect a single image (8). For example, using SDCM, images of microtubules in mitotic cells can be acquired at approximately one second intervals for many minutes with minimal photobleaching (8,11). Another difference in the two types of system is that a SDCM uses a camera as the detector, not a PMT. EMCCD cameras are frequently used for SDCM systems because of their high sensitivity, although the newer CMOS technology allows faster readout and smaller pixels than most EMCCD cameras, thus potentially increasing resolution and speed (12). SDCM systems with ports for more than one camera are also available so that the user can select the camera best suited for the particular experiment. The microscope has optics that can magnify the image from the microscope onto the camera chip, to achieve Nyquist sampling. Although speed is the major advantage of the SDCM, the pinhole size is fixed in the microscope design, so the user cannot vary this parameter. Both LSCM and SDCM systems are available with multiple lasers for imaging a range of fluorescent probes.

A third alternative for imaging mitosis is to use a wide-field fluorescence microscope and perform deconvolution to remove out-of-focus fluorescence (10). Turnkey systems are available for this mode of microscopy (e.g. DeltaVision). The major advantages include the speed of acquisition (no scanning is used) and the fact that a greater percentage of the emitted light is collected, because there is no pinhole. However, images must be processed post-acquisition and depending on the system, this can be slow.

4. Select a suitable cell for imaging. Use phase contrast, DIC or bright field optics. Avoid using fluorescence light, unless absolutely necessary, so as to reduce photobleaching prior to image acquisition (Note ⁴).
5. Set the laser power for each wavelength you will use, and select an exposure time, using the imaging software. Go to live imaging mode, and collect a single image of one fluorophore; adjust focus if needed. Adjust exposure time and/or laser power as if needed so that you can just detect the fluorescent signal(s) of interest. Observe the histogram of pixel intensity distribution and avoid saturating the pixels in the imaging field. Repeat for each fluorophore, as needed.
6. If you are imaging a single Z-plane in the cell, engage the focus control system to automatically maintain focus (Note ⁵).
7. For time-lapse acquisition of a single focal plane, set the interval between images and the duration of the time-lapse sequence. If you want to record the progression through mitosis, use an interval between images that is relatively long to avoid damage to the cell (e.g. 1 image per ~30–60 sec). Using these settings, confirm that the cell progresses through mitosis, and that the fluorescent signal is not photobleached. If better time resolution is needed, try taking images more frequently, again confirming that the cell is not damaged by exposure to the fluorescent light.
8. To acquire a Z-series at a single time point, use the microscope and software controls to set the top and bottom limits for the Z-stack. This is typically performed in live imaging mode. Use a lower exposure time to reduce bleaching as you set up the Z-stack parameters, and re-set the exposure to the desired time for the acquisition. Set the number of Z-planes you wish to collect. Acquire the stack.
9. To acquire a Z-series at multiple time points, set the parameters for Z-image acquisition and the parameters for a time-lapse sequence (Note ⁶).
10. For multi-color imaging, use the software to collect images of multiple fluorophores during a time-lapse sequence or Z-series (Note ⁷).

⁴Photobleaching of the fluorescent molecule of interest is perhaps one of the most frustrating issues in fluorescence microscopy. Possible remedies include using multiple fluorophores (e.g. triple GFP tag, (13)), switching to a fluorophore that is brighter and/or bleaches more slowly (14); using genetically encoded tags that can be modified with a bright organic dye (e.g. Halo tag and Halo ligand). Adjusting the imaging parameters (i.e. exposure time, light intensity) can also help reduce photobleaching; the optimum settings need to be determined empirically for your sample.

⁵During a time-lapse acquisition, the focal plane can change. Typically this results from drift of microscope components due to thermal motion – which is particularly notable when the stage is being heated for live cell imaging. Newer equipment overcomes this issue by holding a set focus using a far red laser beam reflected off the lower surface of the coverslip. In addition to drift resulting from thermal motion, mitotic cells round up to some degree during mitosis. In this case, the structure of interest may go out of focus significantly as the cell changes shape and necessitates changing the focus to keep the structure of interest in focus. Although this can be frustrating, especially during long acquisitions, with practice the user can learn to refocus the cell during the imaging sequence.

⁶Typically, progression through mitosis is adversely impacted if a complete Z-stack is acquired at multiple, consecutive time points. One solution is to collect the fewest number of Z-planes that are required to obtain the necessary information (15), and to increase the time interval between acquisitions. Alternatively, if the information in Z is essential, a complete Z-stack can be taken at different time points, using different cells if necessary.

⁷Collecting multi-color images will expose the mitotic cell to additional light. Using the software, you can acquire images of each fluorophore at each time point, or take fewer images of one (or more) of the fluorophores, depending on the time resolution that is needed for each.

11. Shut down and clean up. Turn off all parts of the system in the order recommended by the manufacturer. Archive data from imaging computer.
12. Clean the objective lens; oil can damage lens elements and must be removed thoroughly at the end of the imaging session (Note ⁸).

PROTOCOL 3: Imaging live mitotic cells with laser scanning confocal microscopy (LSCM)—Follow steps 1–4 from protocol 2.

- 5 Using software controls, set the scan speed and laser power. Acquire a single scan. Adjust parameters so that the fluorescent structure of interest is visible. Observe the histogram of pixel intensity distribution to check for saturated pixels; adjust imaging parameters using the live scan mode so that no pixels are saturated. If necessary, use averaging to decrease noise in the image (Note ⁹).
- 6 Use microscope software to increase the magnification using the zoom feature. Use Nyquist sampling to obtain the theoretical resolution limit for the objective.
- 7 Collect a time-lapse sequence for the duration of mitosis. See Protocol 2, step 7.
- 8 To collect a Z-stack of images, use the microscope software to mark the top and bottom of the region you wish to image. See Protocol 2, step 8. Select the desired number of Z-planes to collect. Acquire the Z-stack.
- 9 Most laser scanning microscopes allow simultaneous acquisition of multiple wavelengths. However, it must be noted that during simultaneous acquisitions, it is possible for the emission signal to bleed over into the red shifted wavelengths and create artifacts. To avoid this, it is best to acquire images sequentially if the fluorophores used overlap in their excitation/emission profiles.
- 10 Shut down and clean up. See Protocol 2, steps 11, 12. Turn off all parts of the system in the order recommended by the manufacturer. Archive data from imaging computer. See Note ⁸.

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⁸Cleaning oil off of the objective lens is particularly important for inverted microscopes and when the microscope is heated. Under these conditions the warm oil will flow down the sides of the objective lens. One solution is to use a fabric-coated elastic band (e.g. ponytail holder) placed around the lens; the fabric absorbs the oil and the elastic secures it to the lens.

⁹We find that to preserve cell health, faster scan time, lower laser power, and 2X averaging work well.

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Figure 1.

Live mitotic LLC-Pk1 cells expressing GFP-tubulin. A. Images acquired using a Laser Scanning confocal microscope, 60 $\times$ objective lens. B. Images acquired using a spinning disc confocal microscope, 100 $\times$ objective lens. A and B are selected images of time series of images acquired at 30 sec intervals. Bars = 10 μ m. Time in min:sec.

Figure 2.

Live LLC-Pk1 cells expressing GFP-tubulin, images acquired using spinning disc confocal microscopy, 100 $\times$ objective lens. A. Astral microtubule dynamics. Left shows single plane of a prometaphase cell; right panels (a–d) show selected images at 15 sec intervals. B. Z-stack of images of a cell with a tilted mitotic spindle. Bottom, middle, and top planes are shown; right panel shows maximum intensity projection of complete Z-series. Bar = 5 μ m in A and 10 μ m in B.