

# Fluorescent Speckle Microscopy (FSM) of Microtubules and Actin in Living Cells

UNIT 4.10

This unit describes the execution of an epifluorescent microscopic method, fluorescent speckle microscopy (FSM), that allows visualization of assembly/disassembly dynamics, movement, and turnover of macromolecular assemblies *in vivo* and *in vitro*. Microinjection or expression in living cells of fluorophore-conjugated proteins followed by their incorporation into cellular structures and visualization by fluorescence microscopy has yielded much information about protein localization and dynamics (Wang, 1989; Prasher, 1995). However, this method has been limited by high-background fluorescence from unincorporated and out-of-focus incorporated fluorescent proteins and difficulty in detecting movement of fluorescent structures because of their uniform labeling. These problems have been partially alleviated by use of more cumbersome methods of laser photobleaching and photoactivation of fluorescence in which a portion of molecules in a structure is marked and the behavior of the molecules in the marked region is monitored (Wolf, 1989; Mitchison et al., 1998). In contrast, FSM uses a very low concentration of fluorescent subunits that co-assemble with endogenous unlabeled subunits giving a structure with a speckled appearance in high-resolution fluorescence images (Waterman-Storer et al., 1998; Fig. 4.10.1). The appearance, disappearance, and movement of speckles stand out to the eye in time-lapse FSM. These changes in fluorescent speckle pattern correspond to the assembly, disassembly, and movement of the structure and can be quantitated (Waterman-Storer et al., 1999). FSM images are captured using conventional wide-field fluorescence light microscopy and digital imaging with a low-noise, cooled charge-coupled device (CCD) camera. FSM provides information about protein dynamics throughout the field of view, as opposed to within a small marked region of the cell as is the case for laser photobleaching and photoactivation of fluorescence techniques. FSM also significantly reduces out-of-focus fluorescence and greatly improves visibility of fluorescently-labeled structures and their dynamics in thick regions of living cells (Waterman-Storer et al., 1998). Finally, the low level of fluorescent protein used in FSM is much less likely to perturb cellular protein balance thereby reducing the chances of artifactual changes in cell behavior due to the effects of protein “overexpression.”

This unit will focus on the utilization of FSM for observing microtubule and actin cytoskeletal dynamics *in vivo*. For microtubules, which can easily be distinguished in fluorescence light microscopy as single filaments both *in vivo* and *in vitro*, the fiduciary marked filaments seen in FSM will allow one to unequivocally distinguish between microtubule translocation as powered by motor proteins and microtubule treadmilling, in which one end of the polymer assembles concomitant with polymer disassembly at the other end (Waterman-Storer and Salmon, 1997). Microtubule FSM also allows observation of microtubule behavior within mitotic spindles containing many hundreds of microtubules and in the central regions of interphase cells. For actin, FSM can be used for studying the assembly dynamics and retrograde movement of actin meshworks in the leading lamella of migrating cells (Waterman-Storer et al., 1998).

## STRATEGIC PLANNING

### General

Successful FSM requires knowledge and experience in high-resolution epifluorescence microscopy, basic tissue culture and biochemistry skills, and considerable skill in single-cell microinjection and handling of cells for live-cell imaging. This unit provides detailed information in basic protocols for designing a digital microscope system for obtaining

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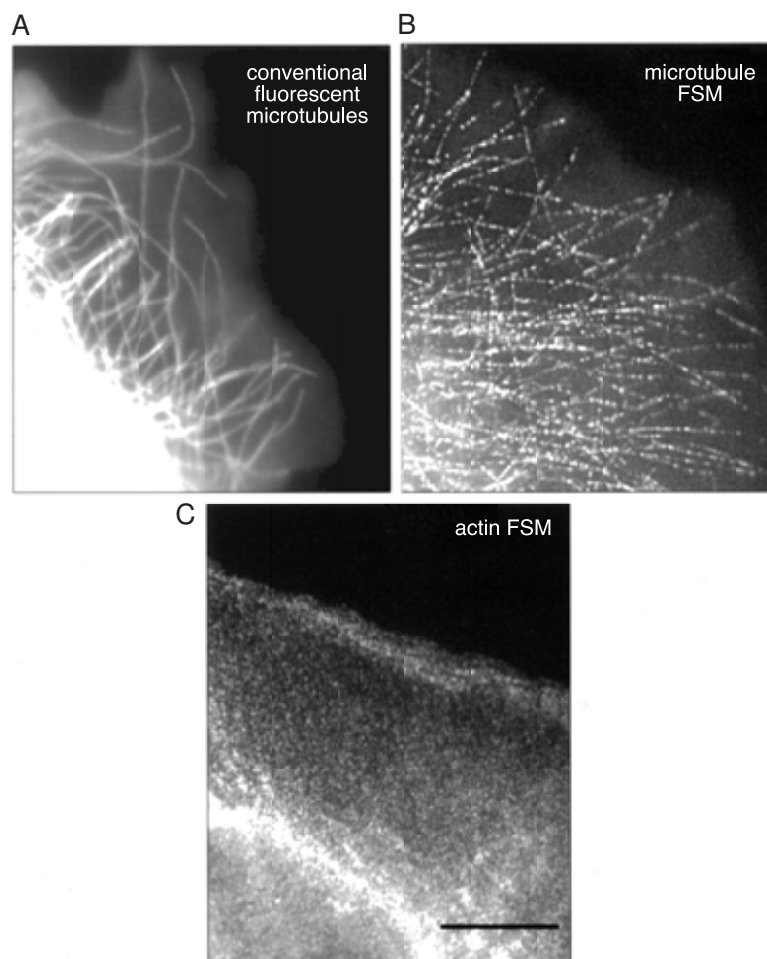
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Microscopy

4.10.1

Supplement 13



**Figure 4.10.1** FSM images of actin (**C**) and microtubules (**B**) in the lamellae of living epithelial cells. A comparison of conventional image of fluorescent microtubules (**A**), labeled with X-rhodamine tubulin, in which labeled tubulin makes up ~10% of the cellular tubulin pool with microtubule FSM (**B**), X-rhodamine tubulin, in which labeled tubulin makes up 0.5% of the tubulin pool. In the conventional image (**A**), there is a high background fluorescence that masks details in the central regions of the cell, and the microtubules are continuously labeled along their lengths, devoid of fiduciary marks. In the FSM image (**B**), the background fluorescence is low, there is no loss in the ability to see the full length of the individual microtubules, and they are covered with fiduciary marks. The images were taken on the same imaging system with the same objective lens. The exposure time for (**A**) was ~200 msec while that for (**B**) was 1500 msec. (**C**) FSM image of a cell injected with low level of X-rhodamine labeled actin. Note the speckled appearance of the lamella, which is made up of a very dense meshwork of cross-linked actin filaments. Bar = 10  $\mu$ m, all images are at the same magnification.

time-lapse FSM images (see Basic Protocol 1), tips for success in live-cell fluorescence microscopy (see Basic Protocol 2), and basic quantitative analysis of FSM images (see Basic Protocol 3); and support protocols for fluorescent labeling of tubulin (see Support Protocol 1) and actin (see Support Protocol 2). Please refer to *UNIT 4.2* for information on basic fluorescence microscopy, *UNIT 1.1* for cell and tissue culturing, and details of how to carry out single-cell microinjection.

Prior to performing FSM imaging, good quality fluorescently labeled protein must be prepared. Labeled proteins that have given good results in FSM applications can be obtained commercially from Cytoskeleton, Inc.. However, if imaging cytoskeletal dynamics is going to be ongoing in the laboratory, it is much more economical to prepare the proteins oneself. For making fluorescent tubulin, unlabeled tubulin must first be made.

Tubulin purification requires a source of fresh pig or cow brains, and can be prepared by the method described in *UNIT 13.1*, Support Protocol 3. Fluorescent labeling of actin requires a source for muscle acetone powder. Although the powder is available from Sigma, better quality acetone powder can be obtained from a laboratory that specializes in actin biochemistry (generally, these laboratories store large quantities in freezers), or alternatively, it can be prepared by the method of Pardee and Spudich (1982).

### Choice of Fluorophore

Support Protocols 1 and 2 describe preparation of tubulin and actin, respectively, that is covalently bound to a succinimidyl ester derivative of the fluorophore of choice. The Molecular Probes catalog is an excellent source of information about the chemistry of fluorophores. Succinimidyl ester derivatives react with lysine residues that are accessible on the surface of the protein. For both tubulin and actin, there is more than one lysine available for reaction, thus, there is the opportunity for higher dye-to-protein ratios, and therefore, brighter fluorescence. For tubulin labeling, succinimidyl esters have, historically, given the greatest success in yield and functional product capable of polymerization and depolymerization (Hyman et al., 1991). Actin labeled with 5-iodoacetamide, which reacts with a single cysteine residue (cys374) on actin, has been well characterized (Wang and Taylor, 1980), however, the dye-to-protein ratio is lower than labeling on lysines.

In choosing the “color” (excitation and emission spectrum) of the fluorophore for FSM imaging, there are two things to keep in mind—the phototoxicity effects on cells of particular wavelengths and endogenous cellular auto-fluorescence. Cells are sensitive to and damaged by short wavelengths; these effects are manifested as retraction of the cell edge, rounding up, and eventually death. Thus, fluorophores that excite at <450 nm (e.g., Coumarin, pyrene) should not be used. Even wavelengths between 450 and 500 nm that excite fluorescein-like dyes (e.g., FITC, Oregon green, Bodipy FL, Alexa 488, or Cy-2) can produce phototoxicity. However, excitation wavelengths beyond the visible (>700 nm, infrared) should be avoided, as well, due to heating effects. The best choice in terms of minimizing phototoxic effects are yellow/orange (570 to 600 nm, tetramethylrhodamine, TAMRA, Cy-3, Bodipy TMR, Alexa 546), orange/red (600 to 630 nm, Lissamine rhodamine, X-rhodamine, Alexa 568, Texas red), or far-red emitting dyes (>630 nm, Cy-5). Of these, the longer wavelength exciters are better, although emission of dyes such as Cy-5 are very difficult, if not impossible, to see by eye, and thus, are problematic to work with. The author’s best success with FSM has come from using the X-rhodamine, Alexa 568, or Texas red fluorophores.

Cellular auto-fluorescence is caused by flavo-proteins that excite in the UV and blue, and emit in blue and green (450 to 550 nm) wavelengths. This auto-fluorescence contributes to background when using fluorescein (FITC) or fluorescein-like dyes, and it “dilutes” the speckle contrast. The author has found this effect to be particularly problematic for actin FSM, in regions of the cell where actin filaments make up cross-linked meshworks that appear as uniform, dense fields of speckles in FSM images (Fig. 4.10.1). If the use of green emitters is unavoidable, as is the case in the author’s laboratory where microtubules and actin are labeled with spectrally distinct fluorophores and monitored by dual wavelength FSM, green emitters can be used with some success for microtubule FSM.

From this discussion, it is apparent that low-level expression of green fluorescent protein (GFP) fusion proteins are not optimal for FSM. First, there is the problem of cellular autofluorescence. Second, the large size (23 kDa) of the GFP moiety, often results in a sizable proportion of the expressed fusion protein losing its ability to functionally incorporate into the cytoskeleton, contributing to background fluorescence. A third problem is the lack of a suitable method for preventing photobleaching of GFP. Finally,

the brightness (quantum efficiency) of the GFP fluorophore is much lower than chemical fluorophores. For studying a microtubule-binding protein, the author has alleviated this last problem to a large degree by fusing multiple (3 to 5) GFP molecules in tandem, joined by short flexible linkers, to the microtubule binding domain of the protein and selecting cells for imaging that express very low levels of the fusion protein (Faire et al., 1999). However, this approach would be unadvisable for actin or tubulin, which would surely be rendered dysfunctional by the incorporation of a 75- to 125-kDa attachment.

## **DESIGNING A MICROSCOPE SYSTEM FOR TIME-LAPSE DIGITAL FSM**

As opposed to a step-by-step protocol, in this section, the basic components needed to set up an FSM system are discussed, giving the recommendations for critical elements required in each type of component (Fig. 4.10.2). It is *imperative* that one reads the Background Information on the theory of FSM image formation before one attempts to understand how and why particular components are chosen for the imaging system.

After reading the discussion of FSM image formation (see Background Information), it is clear that there are three key elements of the microscope imaging system to ensure successful FSM imaging. First, the epifluorescent microscope should be as efficient as possible at collecting photons and should be capable of high-resolution, high-magnification imaging. This is achieved with high-quality, 1.4-NA oil-immersion objective lenses with minimal lens elements. Additionally, this requires a simple optical path between the specimen and camera with as few intervening components as possible. Second, the camera should be highly sensitive, low noise, and should have high enough spatial resolution to match the resolution of the microscope. These properties are met by several cooled CCDs available on the market today. Finally, since fluorophores are subject to photobleaching, light exposure to the specimen should be kept to a minimum. This is accomplished through the use of an electronically controlled shutter on the epi-illuminator that only opens during the time of camera exposure. In addition, a computer with a digital image acquisition board and software for controlling the shutter and image acquisition timing are needed to integrate the system.

### **Materials**

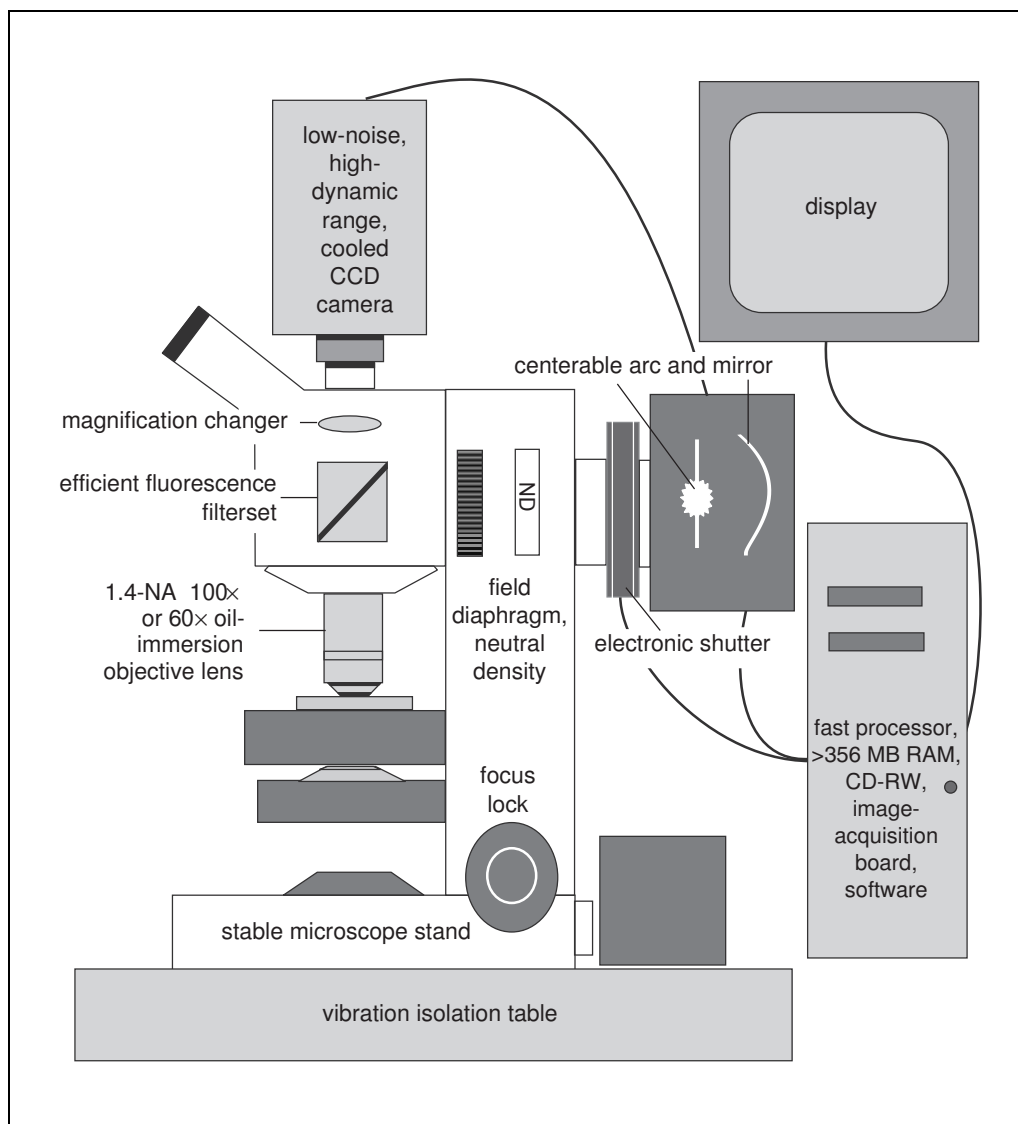
- Upright or inverted epi-fluorescent microscope and optics including:
  - Epi-illuminator
  - High-magnification objective lens (e.g., 60×, 63×, or 100×)
  - Excitation filter, emission filter, and dichromatic mirror
  - Electronically controlled shutter
  - Cooled CCD camera
  - Computer, digital image acquisition board, and software for control of shutter and image acquisition
  - Microscope stand

### ***Upright or inverted epi-fluorescent microscope and optics***

1. The microscope stand should be of biological research quality, with a substantial mass that resists vibration and is not subject to temperature-induced expansion and contraction.

*If possible, the microscope should be mounted on a vibration isolation table. The focusing system should be of high quality, with a means by which to lock focus during long-term time-lapse imaging, since slight shifts in focus result in changes in speckle intensity that can be artifactually interpreted as cytoskeletal dynamics.*

2. The microscope should be equipped with a high quality epi-illuminator including a 100-W HBO mercury arc lamp, and a lamp housing with a parabolic mirror that



**Figure 4.10.2** Diagrammatic view of the components of an upright FSM imaging system. See Basic Protocol 1 for details.

allows manual control of bulb and mirror centration for proper alignment of Köhler epi-illumination.

*It is very helpful, though not necessary, to have manual control of centration and size of the field diaphragm of the epi-illuminator. Closing down the field diaphragm to just the area of the specimen being imaged reduces photo-damage to the whole specimen and reduces out-of-focus fluorescent flare in the image. Similarly, it is helpful if the epi-illuminator is equipped with slots in which to insert neutral density filters to control specimen illumination. An infrared blocking filter can also be included in the illumination path to minimize exposure of the specimen to damaging heat.*

3. The objective lens (which also acts as the condenser in epi-illumination) should allow the highest resolution possible, thus, one with the highest numerical aperture available, i.e., 1.4-NA oil-immersion; and magnification of 60 $\times$ , 63 $\times$ , or 100 $\times$ , the choice being dependent on the spatial resolution of the camera detector (see Matching Microscope and Detector Resolution below).

*Keep in mind that 60 $\times$  or 63 $\times$  lenses are often more efficient at transmitting light than 100 $\times$  lenses. However, never sacrifice system resolution for this small gain in light detection. The*

*lens should not contain contrast-forming elements such as phase rings that block transmission of photons. The lens should be corrected for chromatic aberration (apochromatic) and should be highly efficient at passing the wavelengths of light being imaged, which depends on the choice of fluorophore. Ask the manufacturer for information on the spectral efficiency of the lens.*

*When imaging a small specimen, it is not necessary that the lens be flat-field corrected (plan-corrected). Indeed, often objectives specifically designed for efficient epi-fluorescence (Super Fluor-, Nikon; Fluor-, or Fluar- lenses) are not plan-corrected because this correction adds glass elements to the lens, and the more glass elements, the more places to lose photons. However, for a flat, wide-field view, the author has had good luck using DIC-grade plan apochromat objectives.*

4. The excitation and emission filters and a dichromatic mirror should be as efficient as possible for exciting the fluorophore, separating the excitation from emission, and collecting the emission of the fluorophore of choice. The use of long-pass filters rather than band-pass filters may maximize this efficiency.
5. The path from the objective to the detector should be simple and contain as few intervening components as possible to allow maximum photon collection. Remove analyzers, wave plates, and Wollaston prisms that are used for various modes of polarization microscopy. Optovars or magnification changers should be optimally removed; however, if needed to match the microscope resolution to the detector resolution (see below), this is a source of light loss that will have to be tolerated.
6. The camera port should utilize the most direct path from the specimen. For an upright microscope, this would be the one port directly over the objective, and for an inverted configuration, a bottom or “Keller” port underneath the microscope is best. Remove prisms from the light path that split the image between the ocular and camera port.

*The bottom port requires a hole in the table upon which the microscope is seated, in order to accommodate the camera. If this is not possible, a side camera port, which requires one mirror to direct the image to the camera is better than a front port that requires at least two mirrors.*

#### ***Electronically-controlled shutter***

7. The electronically-controlled shutter should be mounted with proper adapters in the light path between the lamp-house and the epi-illuminator.
8. The shutter should be mirrored on the surface facing the lamp to reflect heat away from the specimen.
9. The shutter should operate quietly, quickly, reliably, and without excessive vibration. Obtain a shutter that can be actuated from a software triggered pulse via the serial or parallel port of the computer.

#### ***Cooled CCD camera***

10. The camera should be a scientific-grade slow-scan cooled CCD camera; a video-rate CCD camera equipped with a cooling device cannot be substituted. The choice of camera will be discussed in terms of the choice of the CCD chip itself and then the electronics of the camera in which the chip is housed. Various combinations of the two are available from different camera manufacturers. For a general introduction and in-depth discussion of CCD cameras, see Inoué and Spring (1997).

#### ***The CCD chip***

11. *Spatial resolution.* The spatial resolution is determined by the physical size of the silicon photodiodes or “pixels” on the CCD chip. These currently range in size from  $\sim 6 \times 6$ – to  $30 \times 30$ – $\mu\text{m}$ . The larger the pixel size, the more magnification from the

microscope will be needed to ensure resolution-limited images. Thus, smaller pixel size is better for FSM as it will not require photon-robbing magnification changers or Optovars in the light path. The total number of pixels making up the CCD and the pixel size will determine the imaging area.

12. *Spectral sensitivity.* Different types of CCD's have specific probabilities at any given wavelength of converting a photon to a photoelectron that is counted as signal by the camera, i.e., quantum efficiency (QE). For example, a Sony HyperHad CCD has ~45% chance of converting a green photon to a photoelectron (45% QE for green light) and a ~25% QE for red light. Thus, this CCD is much more efficient at detecting green fluorescence than red. Manufacturers supply graphs of the wavelength versus QE for their available CCDs. A CCD should be chosen that has spectral sensitivity for the fluorophore of choice.
13. *Illumination geometry.* CCDs can be illuminated from their front or back sides. Illumination from the front requires that the light pass through substrate materials to reach the photosensitive area, reducing QE. Back-illuminated CCDs are physically thinned to allow illumination directly on the back side of the photosensitive surface, making them much more sensitive (also much more fragile and expensive). However, because of the thinning process, there are limits to the size of the pixels, usually around  $\sim 25 \times 25\text{-}\mu\text{m}$ , with the smallest currently available at  $13 \times 13\text{-}\mu\text{m}$ . Thus, one has to weigh whether the increased sensitivity is worth the loss in having to put an Optovar in the image path, as well as whether one can afford the expense. For FSM applications, sensitive front-illuminated CCDs have worked quite well.
14. *Readout geometry.* Once photons are converted to charge in the array of pixels, the charges must be read out to an image acquisition board so that the image can be reconstructed in the computer by assigning a gray value to the relative charge at each pixel position. Charges are transferred out of the CCD in three basic ways. Full-frame readout occurs as each row of pixel charges is transferred serially out of the CCD one row after another. This type is the slowest and introduces the most noise into the image, although, this type of readout is still acceptable for FSM if other camera electronics do not introduce sources of noise. In contrast, in frame-transfer and interline-transfer CCDs, either the entire pixel charge array or whole rows of pixels are rapidly transferred to an array of pixels that are masked from light. The charges are then read out from the masked area while the imaging area is being exposed to light again. These types are much faster and less noisy than the full-frame readout. Frame-transfer CCDs tend to be more expensive and interline-transfer CCDs suffer from lower resolution, although this problem has been overcome in recent years. Thus, all three geometries are acceptable for FSM, although the increased speed of the interline-transfer CCD may offer an advantage for increasing image-acquisition rate to observe actively ruffling cells or microtubule motor activity.

#### **Camera electronics**

15. A CCD with the lowest temperature cooling system within the means of one's budget should be chosen. Heat on the CCD can be interpreted by the photodiodes as light, thus, contributing to image noise. Different camera manufacturers will house the same CCD in cameras with different degrees of cooling ranging from 20°C below room temperature to -50° or -60°C. Do not choose the least expensive CCD because heat is an avoidable source for noise that can easily mask the very faint FSM signal.
16. *Readout speed.* In general, the faster the readout speed, the more error is introduced during charge transfer, which translates to noise in the image. Speeds in modern cameras range from 100 kHz in some low-noise back-illuminated cameras, to 14 to

15 MHz in interline- and frame-transfer cameras. For quantitative FSM imaging of cytoskeletal dynamics, image acquisition rates of 1 to 2 images/sec may be required, which cannot be accomplished by the slower cameras. Here, a reasonable compromise of speed and low noise must be sought, but it is recommended not to buy a camera much slower than 1 mHz.

17. *Dynamic range.* Although the total number of photons that can be absorbed by each pixel before it is saturated with charge is set for a given CCD, the number of gray levels this amount of charge is divided up into for display is not fixed. It can be represented by 8, 10, 12, 14, or 16 bits of information per pixel, corresponding to 256, 1024, 4096, 16,384, or 65,536 ( $2^8$ ,  $2^{10}$ ,  $2^{12}$ ,  $2^{14}$ , or  $2^{16}$ ) gray levels, respectively. For FSM imaging of very dim specimens, it is important to have the biggest dynamic range possible (again, within a reasonable budget). This is required so that there will be finer gradations of gray within the very small portion of the dynamic range that is utilized. Successful FSM has been achieved using 10-bit cameras, although  $\geq 12$  bits/pixel is recommended.
18. *Subarraying and binning.* Being able to read out only a specified portion of the CCD (subarraying) can increase image acquisition speed for imaging small areas of a cell, but it is not necessary. Binning, in which the charges in a group of pixels are combined and read out as a single pixel to increase sensitivity should not be done in FSM, as this effectively increases pixel size and decreases CCD resolution.

***Computer, digital image acquisition board, and software for control of shutter and image acquisition***

19. A computer with the fastest processor and most random access memory (RAM) affordable should be used. Time-lapse FSM image series are large files often on the order of  $\geq 100$  MB, and computer “horsepower” is necessary to view and manipulate these.

*The author recommends  $\geq 356$  MB of RAM.*

20. *File storage.* A compact disc read/write (CD R/W) device is the most economical choice recommended to archive the large files generated by time-lapse FSM. The fastest write speed available within budget should be chosen.
21. Use the image acquisition board recommended by the camera and software manufacturer, making sure that the board can handle the bit depth of the camera. Many cameras come with their own boards.
22. Software should be capable of time-lapse digital image acquisition and triggering the shutter during camera exposure. The software should allow easy viewing of time-lapse series as movies, with control of play-back rate and adjustment of brightness and contrast in the entire image series. Basic image processing including the ability to perform low-pass filtering and image arithmetic (subtraction, multiplication, etc.) is required. The software should provide the ability to perform quantitative analysis of intensity, position, and distance.

*The author has used MetaMorph (Universal Imaging) with outstanding success. However, NIH-Image freeware (<http://rsb.info.nih.gov/nih-image/>) is also very versatile and many free macros are available.*

***Matching microscope and detector resolution***

23. The key to actually achieving resolution-limited images of fluorescent speckles is matching microscope and detector resolution. Magnifying the diffraction-limited spot to the size of 3 pixels on the CCD is required so that the CCD does not limit



imaging system resolution or produce aliasing between pixels. This is called the Nyquist sampling criterion. Any magnification over this value does not contain any more information and simply reduces the area of the specimen that is imaged. The magnification ( $M$ ) required to achieve this is given by

$$M = 3(P_{\text{width}})/r$$

where  $P_{\text{width}}$  is the width of a pixel on the CCD and  $r$  is the size of the diffraction-limited spot. Thus, for red fluorescence with a resolution limit of 0.27  $\mu\text{m}$  (for red fluorescence, see Background Information) and a camera with 6.7- $\mu\text{m}$  pixels, the magnification required to satisfy the Nyquist criterion is 74.4 $\times$ . Thus, either a 100 $\times$  objective or a 60 $\times$  with a 1.25 $\times$  Optovar should be used, whichever transmits more light.

## TIME-LAPSE FSM IMAGING OF THE CYTOSKELETON IN LIVING CELLS

This protocol describes how to handle living cells for FSM imaging. Included are basic recommendations for microinjection, detailed instructions for prevention of photobleaching, and image acquisition. Photobleaching of chemical fluorophores (not GFP) is dependent on dissolved oxygen in the medium. Thus, photobleaching can be substantially prevented by removal of dissolved oxygen, in this case, with a commercial product, Oxyrase, and by then sealing the imaging chamber from contact with air (Waterman-Storer et al., 1993).

### Materials

- ~0.5 mg/ml labeled cytoskeletal protein (tubulin or actin, see Support Protocols 1 or 2, respectively)
- Cultured tissue cells grown on 22  $\times$  22-mm glass coverslips in small plastic petri dishes
- Valap (UNIT 13.1)
- Buffered filming medium (see recipe)
- Oxyrase EC (Oxyrase Inc.)
- Microultracentrifuge (Optima TL or TLX, Beckman Instruments; or Discovery M120, Sorvall)
- 0.6-ml microcentrifuge tubes
- Swinging bucket rotor for microultracentrifuge with adapters for holding 0.6-ml microcentrifuge tubes (for the Beckman TLS-55 rotor, the standard thick-walled 1.4-ml polycarbonate tubes are used; and for the Sorvall, custom adapters are needed)
- Microinjection needles (see recipe)
- Microloader pipet tips (Eppendorf or equivalent) or narrow-gauge syringe (narrow enough to fit in the bore of the microinjection needle, Hamilton Company)
- Single-cell microinjection system capable of controlled backpressure of 0.1 to 20 psi and with a precision micromanipulator for injection of single cells, mounted on an inverted microscope equipped with a long-working-distance phase-contrast condenser and a 40 $\times$  dry phase-contrast objective lens with a working distance long enough to focus through the plastic petri dish and coverslip.
- Microscope stage incubator (optional)
- 1  $\times$  3-in. glass microscope slides
- Scotch double-stick tape (3M)
- Cotton swabs
- FSM imaging system (see Basic Protocol 1)

## BASIC PROTOCOL 2

### Microscopy

#### 4.10.9

### ***Prepare labeled cytoskeletal protein***

1. Cool the microultracentrifuge and rotor to 4°C.
2. Rapidly thaw and immediately place on ice a 3- $\mu$ l aliquot of ~0.5 mg/ml labeled cytoskeletal protein.
3. Pellet insoluble protein in the sample. Cut off the lid of the 0.6-ml microcentrifuge tube containing the labeled protein and place the tube in the polycarbonate adapter for the swinging bucket rotor. Make sure to place a balance tube in the opposite bucket. Microultracentrifuge 20 min at  $77,000 \times g$  (Beckman TLS-55 rotor at 30,000 rpm), 4°C.

*Do not microultracentrifuge any faster, as this will crush the microcentrifuge tube.*

4. During the microultracentrifugation, locate the cultured tissue cells to be injected on the microscope stage using a 40 $\times$  phase-contrast objective lens.

*All manufacturers correct their high-resolution optics for the thickness of a no. 1.5 coverslip.*

*Cell choice is obviously dependent on the biological question; however, it is advisable to choose flat, large, well-spread cells as these are better for microinjecting and imaging. PtK1 are a favorite for mitosis studies, while Swiss 3T3 are a favorite for cell locomotion studies.*

5. Following microultracentrifugation, promptly remove the sample from the microultracentrifuge and transfer to ice.

### ***Microinject cells with labeled protein***

6. Backload a microinjection needle with ~0.5  $\mu$ l of protein solution using either a microloader pipet tip or a narrow-gauge syringe.

*Be careful not to touch the insoluble pellet when retrieving the solution from the microcentrifuge tube.*

7. Microinject several cells in the center of the coverslip using ~0.5 to 1.5 psi constant pressure.

*The constant flow from the needle helps to keep it from clogging.*

*It is not recommended to transiently increase the pressure during microinjection, as this will result in too much fluorescent protein in the cells, ruining the speckle effect.*

8. Return the cells to a 37°C, 5% CO<sub>2</sub> incubator for 1 hr to recover from microinjection and to allow incorporation of labeled protein into the cytoskeleton.
9. During this time, place filming medium in a 37°C water bath, melt Valap on a hot plate set on low; and turn on the microscope, camera, and stage incubator to allow temperatures to equilibrate.

*A microscope stage incubator is only required for cells from warm-blooded species. An incubator can be as simple as a home hair blow dryer custom-fitted with a rheostat for control of the heating coils and a thermometer taped to the stage.*

### ***Mount cells for imaging***

10. Just prior to mounting the specimen, prepare 1 ml of buffered filming medium containing 20 to 30  $\mu$ l Oxyrase EC.

*The amount of Oxyrase the specific cell type can tolerate must be determined empirically. Some cells are more sensitive to oxygen deprivation than others. For sensitive cells (such as PtK1), make sure the filming medium is prepared with high glucose medium, so that extra glucose will be available for anaerobic metabolism.*

11. Prepare a filming chamber by placing two  $3 \times 30$ -mm strips of double-stick tape side by side 1 cm apart and parallel with the long axis onto a  $1 \times 3$ -in. glass microscope slide to form a channel where the cells will be.
12. Retrieve the coverslip containing the injected cells from the dish of medium, dry the back side and the edges that will be placed on the tape strips, and place the coverslip, cell-side down onto the tape so that the area containing the injected cells lies between the strips of tape.

*Press the coverslip into place to form a good seal with the tape.*

13. Add Oxyrase-containing filming medium to the cell “chamber” by pipetting it against the edge of the coverslip between the strips of tape. Exchange the medium several times by wicking away medium with filter paper from the opposite open end of the chamber while pipetting.

*Be sure not to introduce any bubbles into the chamber during pipetting as the presence of air in the chamber will cause problems with photobleaching.*

14. Carefully dry the edges of the specimen chamber (do not introduce bubbles) and use a cotton swab to apply melted Valap along all edges of the coverslip to seal it completely to the slide.

#### **Observe mounted cells**

15. Allow the slide to equilibrate to the proper temperature on the microscope stage for 10 to 15 min. Turn the room lights off to allow one’s eyes to adapt to the dark.
16. Use epifluorescent illumination to locate the fluorescent-injected cells.

*The cells should be quite dimly fluorescent and may not be visible with a low-power, low-NA objective lens, therefore, one may have to search with an oil-immersion lens. After locating the cells, switch to high-magnification, high-NA objective lens. At this point, be careful about illuminating the specimen and causing photobleaching—look only as long as needed to get a cell in focus and centered in the field of view then quickly shutter the light. Use of neutral density filters to attenuate illumination during focus and centration may also be helpful in preserving the fluorescence in the specimen.*

*Cytoskeletal polymers are excluded from the nucleus; cells with fluorescent nuclei are likely dead. Dimly fluorescent individual microtubules should be visible by eye, while fluorescent actin should be rather diffuse throughout the cell and perhaps brighter in stress fibers.*

17. Pick a dim cell to start with because microinjection will result in cells of varying fluorescent brightness.

*With practice, one will be able to judge by eye the brightness required for good FSM.*

18. Illuminate only the area of interest by adjusting the field diaphragm of the epi-illuminator.

#### **Optimize camera conditions and acquire time-lapse images**

19. Optimize exposure time. First, make sure the camera is set to the maximal dynamic range possible and set the image display to “autoscale.” Without autoscaling, the image will likely appear black since the FSM image is expected to use a very small portion of the low end of the dynamic range of the camera. Then, take a 500-msec “dark image” image with the illuminating light shuttered to get a measurement of the background gray level of the camera. Next, be sure the illumination shutter is set to synchronize with the camera exposure. Then, take images of the fully illuminated specimen (no neutral density filters), trying to get the specimen signal to ~10% higher in gray level value than the background.

For example, if the background gray level in a 14-bit (16,384 gray level) camera is 500, try for an exposure that will give the specimen a gray level of ~550. The sample should be dim enough that this very small amount of signal above the noise should require a rather long exposure time. A good starting point is 500- to 1000-msec exposures, but upwards of 2000 msec may be required.

The time of exposure will vary depending on the camera used, the brightness of the sample, the age of the arc lamp, and the tolerance of the specimen to light. Signs of photo-damage include cessation of cell motility, retraction of cell lamellipodia, or blockage of mitotic progression.

20. Optimize focus. Take exposures while slightly adjusting the fine focus until the best focused-fluorescent speckles can be seen. Then, lock the focus.

*If the actin network does not appear speckly, or if individual microtubules do not appear discontinuously labeled along their lengths, there is too much labeled protein in the cell. Find another, more dimly labeled cell.*

21. Acquire time-lapse image series. For following actin movement, acquire images every 10 to 30 sec. For following microtubule dynamics and movement, acquire images at 3- to 10-sec intervals. Image as long as is needed or until the sample is photobleached.

### BASIC PROTOCOL 3

## QUALITATIVE AND QUANTITATIVE ANALYSIS OF TIME-LAPSE FSM IMAGE SERIES

Methods are presented to extract measurements of speckle movement and lifetime to determine polymer velocities and turnover times.

### Materials

Stage micrometer (Fisher)  
Image analysis software

1. Calibrate the pixel-to-distance conversion factor. Take an image of a 10- $\mu$ m stage micrometer using the objective lens used for image acquisition.

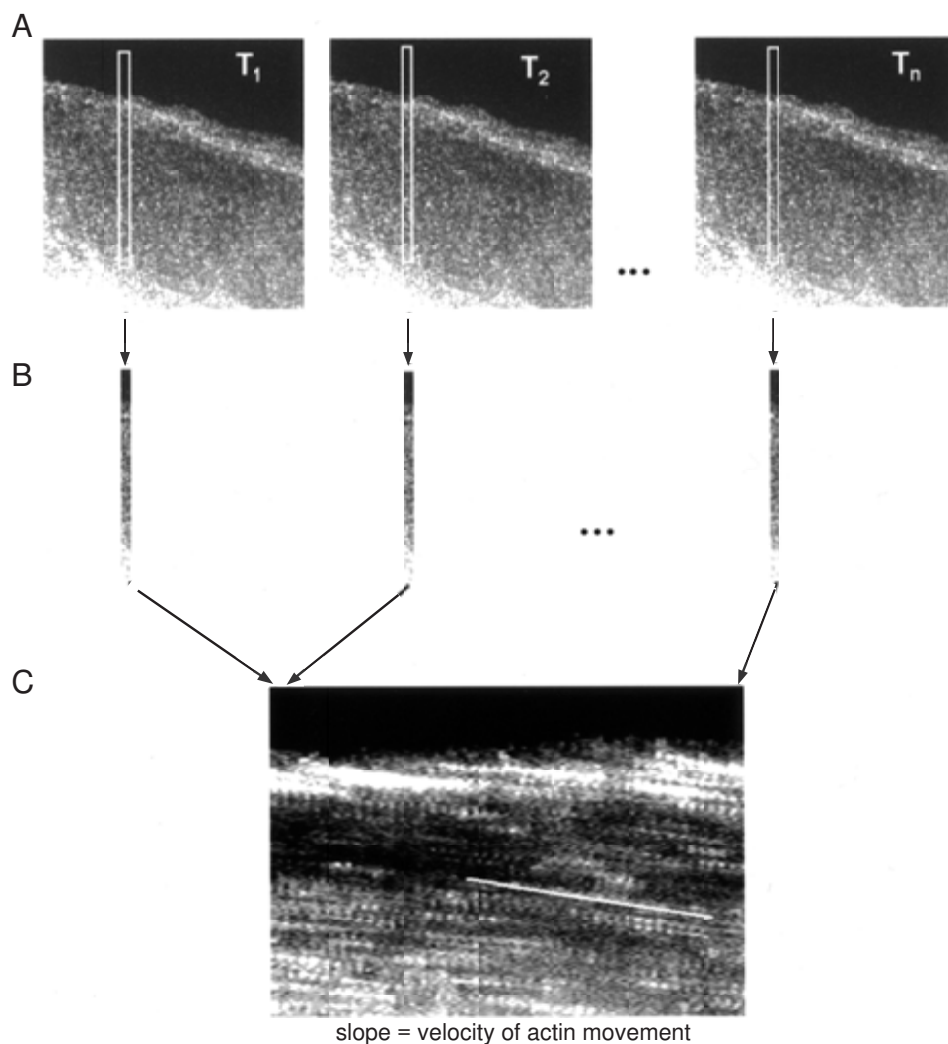
*The number of pixels in 10- $\mu$ m is used to calculate the pixel-to-distance conversion factor. Most software packages will allow this factor to be input so that measurements made are automatically reported in micrometers as opposed to pixels.*

2. Adjust the brightness and contrast of the image series to maximize the speckle appearance.
3. Play the time-lapse series as a movie, paying attention to the movement, appearance, and disappearance of fluorescent speckles.
4. To enhance FSM images, perform an “unsharp mask” filter on each image in the time-lapse series (Waterman-Storer et al., 1999).

*This consists of performing a  $9 \times 9$ - or  $10 \times 10$ -low-pass filter on the image and then multiplying by a constant of 0.5. This low-passed, scaled image is then subtracted from the original image, and the result is multiplied by a constant to scale it to the same average gray level as the original image. This series of operations greatly enhances speckle appearance.*

5. To measure speckle movement rates in complex fields of speckles such as the mitotic spindle or the actin-rich lamella of a migrating cell, perform kymograph analysis (Waterman-Storer et al., 1999; Fig. 4.10.3).

*There are two ways to make kymographs of speckle motion in an image series. In the first method, a narrow rectangular cursor is drawn on the image stack, with the long axis of the*



**Figure 4.10.3** Construction of a kymograph. **(A)** A long thin rectangular region whose long axis is parallel to the direction of speckle movement is selected in each image in a time-lapse series ( $T_1$ -  $T_n$ ). **(B)** This region is extracted from each image and pasted side-by-side in a montage to form the kymograph **(C)**. As speckles move along the axis of the rectangular region in the original image series over time, they appear as oblique streaks on the kymograph, the slope of which correspond to the velocity of speckle movement (see Basic Protocol 3).

*rectangle aligned with the direction of speckle movement determined by viewing the series as a movie. The rectangle should be 2 to 4 pixels in width and the length of the area desired for motion analysis. This region of interest is then copied from each image in the time-lapse series, and pasted side-by-side in a montage to form the kymograph picture. By eye, the speckles within the rectangular region that are moving along its long axis produce oblique white lines in the kymograph, the slope of which yields the velocity of motion. The disadvantage of this method is that it only measures speckle movements in either horizontal or vertical directions.*

*The second method requires the image processing software to possess a "kymograph function." The MetaMorph system is used as an example. The point-to-point line drawing function in MetaMorph is used to draw a line in any direction along the direction of movement for motion analysis on the first image in the time-lapse series. This has the advantage that the line can curve with the curvature of motion. The "kymograph" routine in MetaMorph samples pixel intensity values along this line for each image in a series and*

montages these values into an image where the horizontal direction is the pixel values measured along the line, and the vertical direction is lines from subsequent images in the time series. Measurements of distance in the horizontal direction are calibrated in micrometer units. Measurements of distance in the vertical direction must be calibrated to the total time of the number of images in the series. For example, if the images are recorded at 10-sec intervals and there are 50 images in the series, then the vertical distance of the kymograph corresponds to 500 sec. Velocities are determined by the slopes of the oblique trajectories of speckle movements in the kymographs.

6. Use single-speckle motion tracking to track the movement of single cytoskeletal polymers.

*Currently, this can only be done by hand, but software for automated tracking is being developed. Commercially available automated tracking software is incapable of tracking single speckles in a dense field of similar speckles, which in addition are often fuzzy, ill-defined, tiny, and subject to intensity changes due to slight shifts in focus. Hand tracking is done by measuring the distance of the speckle from its origin at time zero in each frame of the time-lapse series. This can often be done semi-automatically using software functions such as "track points" in MetaMorph. The distance over time can be used to calculate instantaneous velocities (change in distance from frame  $n$  to frame  $n + 1$ ) or average velocities by performing regression analysis of the distance versus time data.*

7. Measure speckle lifetime to give a value for the lifetime of the cytoskeletal filament, i.e., its rate of turnover.

*In theory, the intensity value of a single pixel at the center of a fluorescent speckle could be recorded at every point in time and the lifetime of the speckle determined from this "fluorescent life history." However, this is currently very difficult in practice because of problems with photobleaching, shifts in focus that affect speckle intensity, and minute movement of speckles in living specimens. Again, image analysis software that takes these problems into consideration is currently being developed.*

## SUPPORT PROTOCOL 1

### PREPARATION OF FLUORESCENTLY LABELED TUBULIN FOR FSM

This is a modification of a protocol developed by Hyman et al. (1991; also see the Mitchison laboratory Web page, <http://iccbweb.med.harvard.edu/mitchisonlab/Pages/label.html>). In this method, tubulin is first polymerized to bury the sites of tubulin dimer-dimer interactions to mask them from being labeled, then polymerized tubulin is reacted with fluorophore. The succinimidyl ester derivative of the fluorescent dye reacts with lysine residues at a high pH. Tubulin is readily denatured at high pH, so the longer the labeling reaction is allowed to proceed, the lower the yield, therefore, do not exceed the recommended reaction time. The reaction is then quenched by lowering the pH, and the tubulin is cycled for a temperature-dependent round of depolymerization and repolymerization (see UNIT 13.1 for more information on the biochemistry of tubulin) to select for the assembly competent labeled tubulin dimers before finally being depolymerized in microinjection buffer and frozen for storage. This method takes ~7 hr and should yield ~10 mg of labeled tubulin, which should be useful for 6 to 8 months frozen at  $-80^{\circ}\text{C}$ .

#### Materials

10-ml aliquots of phosphocellulose-purified tubulin in column buffer (CB; totaling 40 to 60 mg of tubulin; UNIT 13.1)  
CB/BRB-80 conversion buffer (see recipe)  
100 mM GTP (see recipe)  
Glycerol  
Labeling buffer (see recipe)  
High-pH cushion (see recipe)  
Quench (see recipe)

Low-pH cushion (see recipe)  
 Succinimidyl-ester derivative of fluorescent probe of choice  
 Anhydrous DMSO  
 Injection buffer (IB; see recipe)  
 1× BRB-80 (see recipe for 10×)  
 1 M MgCl<sub>2</sub> (APPENDIX 2A)  
 37°C water bath  
 100- and 10-ml graduated cylinder  
 Parafilm  
 Ultracentrifuge (with a Beckman 70.1 Ti rotor; or equivalent)  
 13.5-ml ultracentrifuge screw-cap polycarbonate tubes  
 Manual pipet-pump type pipettor (VWR or equivalent)  
 1.6-ml microcentrifuge tubes  
 Microultracentrifuge (Beckman Optima TLX with TLA-100.4 rotor or Sorvall RC M120 GX with S100AT4 rotor)  
 5.1-ml microultracentrifuge polycarbonate tubes  
 7-ml dounce homogenizer with “B” pestle  
 Spectrophotometer with a small-volume quartz cuvette  
 0.6-ml UV-impermeant microcentrifuge tubes

### ***Prepare tubulin***

1. Thaw three to four 10-ml aliquots of phosphocellulose-purified tubulin (frozen off the column in CB; UNIT 13.1) by immersing tubes in a 37°C water bath and agitating gently and continuously until they are fully thawed. As soon as the solutions are fully thawed, transfer tubes to ice and pool the aliquots into a 100-ml graduated cylinder on ice.
2. Add 1/20 vol CB/BRB-80 conversion buffer and add 100 mM GTP for a final concentration of 1 mM. Allow tubulin to bind GTP for 5 min on ice.
3. Add 1/3 vol glycerol, cover the cylinder with Parafilm, and mix very well by gentle inversion.
4. Incubate 40 min in a 37°C water bath to allow tubulin to polymerize, gently mixing every 10 min.

*Make sure the water level in the bath is as deep as the liquid level in the cylinder so that the temperature is constant throughout the whole solution.*

5. During this incubation prewarm the ultracentrifuge with a Beckman 70.1 Ti rotor, labeling buffer, high-pH cushion, Quench, low-pH cushion, and several 13.5-ml ultracentrifuge screw-cap polycarbonate tubes to 37°C.

*The rotor can be prewarmed by putting it in a watertight plastic bag and submersing it in warm water (in a separate 37°C water bath from the polymerization reaction). The centrifuge may only warm up to ambient temperature. If this is the case, so long as the rotor is at the proper temperature, the microtubules should be intact.*

### ***Isolate microtubules***

6. At the end of the incubation, add 3 ml warm high-pH cushion to each warm 13.5-ml ultracentrifuge tube. Slowly layer the polymerized tubulin onto the cushion by using a manual pipet-pump to slowly drip the tubulin solution down the side of the ultracentrifuge tube, being careful not to mix the tubulin with the dense cushion. Divide the microtubule solution evenly among the ultracentrifuge tubes. Balance pairs of filled ultracentrifuge tubes to within 0.01 g, and remember to include the centrifuge cap.

7. Ultracentrifuge the microtubules 1 hr at  $185,000 \times g$  (Beckman 70.1 Ti rotor at 52,000 rpm),  $37^{\circ}\text{C}$ .
8. During the ultracentrifugation, make up 100 mM succinimidyl-ester derivative of fluorescent dye of choice in anhydrous DMSO in a 1.6-ml microcentrifuge tube. Warm to  $37^{\circ}\text{C}$  and vortex it well to get as much as possible into solution.
9. Just before microtubule ultracentrifugation is over (step 7), microcentrifuge the insoluble fluorescent dye solution for a few minutes at maximum speed, room temperature. Promptly collect the supernatant and transfer it to a new 1.6-ml microcentrifuge tube.
10. After the ultracentrifugation, note the position of the clear/pearlescent pellet in each tube. Aspirate the supernatant to half the height of the cushion, and wash the cushion with 1 to 2 ml of warm labeling buffer. Then, completely aspirate the supernatant off, rinse the pellet gently with 1 ml warm labeling buffer, and aspirate again. Immediately add 500  $\mu\text{l}$  warm labeling buffer to each tube, and return the tubes to the  $37^{\circ}\text{C}$  water bath.
11. Resuspend each pellet by pipetting up and down in warm labeling buffer. While resuspending one pellet, keep the remaining tubes in the  $37^{\circ}\text{C}$  water bath, and use the same pipet tip for all tubes.

*Careful and thorough resuspension is key to high labeling efficiency.*

*The pellets are dense and sticky. Use a cut-off tip (cut ~5 mm off the end) on a 1-ml pipetman, starting with the 500  $\mu\text{l}$  in each tube. Dial the pipetman in to 300  $\mu\text{l}$ , and pipet liquid up and down over the pellet until it is fully resuspended with no chunks present. Be patient, avoid frothing the solution, and do not poke the tip into the pellet as chunks of the pellet will get stuck inside the tip and will be lost.*

12. After the first 500  $\mu\text{l}$ , pool the resuspended pellets in one tube in the  $37^{\circ}\text{C}$  water bath, and go back and rinse each tube thoroughly with another 300  $\mu\text{l}$  of warm labeling buffer. Pool all together, a total of 2 to 3 ml, and keep in the  $37^{\circ}\text{C}$  water bath.

#### ***Label microtubules***

13. Add fluorophore dye solution (step 9) to the resuspended microtubules to 10 mM final concentration, mix well by gentle vortexing, and incubate 30 min at  $37^{\circ}\text{C}$ . Gently vortex every few minutes throughout the labeling reaction, being careful not to froth the solution.
14. During the labeling incubation, warm up the microultracentrifuge with rotor and several 5.1-ml microultracentrifuge polycarbonate tubes to  $37^{\circ}\text{C}$ .
15. Stop the labeling reaction by adding an equal volume of Quench.
16. Put 1 ml of low-pH cushion into each 5.1-ml microultracentrifuge tube, and layer 2 ml of the labeled polymer on top. Microultracentrifuge microtubules 30 min at  $200,000 \times g$  (Beckman TLA-100.4 rotor at 70,000 rpm),  $37^{\circ}\text{C}$ .
17. During the centrifugation, cool a 7-ml dounce homogenizer, pestle, and 10 ml of injection buffer (IB) on ice and warm up a few milliliters of distilled water to  $37^{\circ}\text{C}$ .
18. After centrifugation, aspirate the supernatant to 1/2 cushion volume, wash the cushion with a few drops of warm distilled water, and then aspirate the remaining cushion.

#### ***Depolymerize labeled microtubules***

19. Immediately add 500  $\mu\text{l}$  cold IB to each tube. Incubate 10 min on ice to let depolymerization initiate before beginning to resuspend the pellets. Pool resuspended



pellets into the dounce homogenizer on ice, rinse each tube with an additional 200  $\mu$ l of cold IB, pool all.

*This pellet will be horribly sticky. Do not attempt to fully resuspend the pellet in the microultracentrifuge tube, but instead try to partially resuspend as much solids as possible and transfer them into the homogenizer. As always, be patient and do not introduce bubbles into the solution.*

20. Allow the microtubules to depolymerize for 30 min on ice, slowly raising and lowering the pestel in the homogenizer the entire time, being careful not to introduce bubbles and keeping the homogenizer immersed in ice.
21. During depolymerization, chill the microultracentrifuge, a few 5.1-ml microultracentrifuge tubes, and the rotor to 2°C.
22. Transfer the resuspended pellets to cold 5.1-ml microultracentrifuge tubes and clarify the depolymerized tubulin by microultracentrifuging 10 min at  $415,000 \times g$  (Beckman TLA-100.4 rotor at 100,000 rpm), 2°C.

#### ***Polymerize labeled microtubules***

23. Collect the supernatants and pool in a 10-ml graduated cylinder at room temperature. Add 1/10 vol of 10 $\times$  BRB-80, 1 M  $MgCl_2$  to a final concentration of 4 mM, 100 mM GTP to a final concentration of 1 mM, and finally 1/3 vol glycerol. Mix well by inversion.
24. Polymerize microtubules by incubating the graduated cylinder, covered with Parafilm, for 45 min in a 37°C water bath, with occasional mixing.
25. During the incubation, warm up the rotor, microultracentrifuge, and two 5.1-ml microultracentrifuge tubes to 37°C.
26. Just prior to termination of incubation, put 0.5 ml of low-pH cushion in each warm 5.1-ml microultracentrifuge tube. After the incubation, layer the polymerized microtubules on each low-pH cushion.
27. Microultracentrifuge microtubules 30 min at  $200,000 \times g$  (in the TLA-100.4 rotor at 70,000 rpm), 37°C.
28. During this microultracentrifugation, chill 5 ml IB on ice and warm 5 ml of 1 $\times$  BRB-80 to 37°C.
29. After the microultracentrifugation, aspirate the supernatant to 1/2 cushion volume, wash the cushion two times with a few drops of warm 1 $\times$  BRB-80, then aspirate the supernatant completely, wash the pellet very gently two times with warm 1 $\times$  BRB-80 to remove all residual glycerol (which is toxic if microinjected into cells).
30. Cover pellets with 200  $\mu$ l of cold IB and incubate 5 min on ice before resuspending the pellets. During this time, chill the microultracentrifuge and rotor to 4°C.
31. Resuspend the pellets in a total of 300  $\mu$ l per tube by pipetting, using the second 100  $\mu$ l as a rinse. Pool the resuspended pellets in a clean, chilled microultracentrifuge tube and cover it with Parafilm.

*These pellets should not be as sticky as the last ones and should be fairly easy to resuspend by pipetting.*

#### ***Depolymerize microtubules***

32. Depolymerize the resuspended microtubules by incubating 30 min on ice.

33. Clarify the depolymerized microtubules by microultracentrifuging for 5 min at  $415,000 \times g$  (in the TLA-100.4 rotor at 100,000 rpm), 4°C.
34. After microultracentrifugation, transfer the supernatant to a new 1.6-ml microcentrifuge tube on ice.

***Calculate concentration of tubulin and fluorophore***

35. Calculate concentration of tubulin, the fluorescent dye-to-protein ratio, and the percent yield of the preparation. Measure the absorbance of a 1:100 dilution (in water) of the tubulin solution at 278 nm ( $A_{278}$ ) and at the excitation maximum of the fluorophore in a quartz cuvette, using water to blank the spectrophotometer. Calculate the concentration of tubulin using the extinction coefficient for tubulin  $\epsilon = 115,000 \text{ M}^{-1} \text{ cm}^{-1}$  at 278 nm and Beer's law:

$$\text{Concentration} = \text{dilution factor} \times A_{278}/\epsilon$$

36. Calculate the concentration of fluorescent dye similarly, using the extinction coefficient supplied by the fluorescent dye manufacturer (contact technical service for information).

*The fluorescent dye-to-protein ratio is simply the ratio of the molar concentrations. For calculation of the percent yield, determine the total amount of labeled tubulin obtained from the concentration and volume and use the molecular weight of the dimer, 110 kDa to convert to milligrams to compare to the original amount of tubulin labeled.*

37. Adjust the concentration of labeled tubulin to 20 to 30 mg/ml with IB, and drop freeze 3- $\mu$ l aliquots in 0.6-ml UV-impermeant microcentrifuge tubes in liquid nitrogen for storage at  $-80^{\circ}\text{C}$ .
38. For use for microinjection, rapidly thaw an aliquot of concentrated labeled tubulin and transfer immediately to ice. Dilute to 0.5 mg/ml with cold IB, and drop freeze 3- $\mu$ l aliquots for use within 2 weeks.

**SUPPORT  
PROTOCOL 2**

**PREPARATION OF FLUORESCENTLY LABELED ACTIN FOR FSM**

This is a modification of a protocol developed by J.S. Sanger and J.W. Sanger (Turnacioglu et al., 1998). In this protocol, actin is labeled on lysines with a succinimidyl ester derivative of the fluorescent dye of choice. The labeling is carried out on actin filaments, so that the dye does not bind to regions of the actin monomer that are involved in filament formation, and then the labeled actin is taken through rounds of depolymerization, polymerization, and depolymerization to select for functional labeled molecules, similar to the protocol for labeling tubulin. However, actin polymerization is not temperature dependent like tubulin, but salt dependent with polymerization driven by high-salt concentrations and depolymerization occurring at low-salt concentrations (Pardee and Spudich, 1982). Thus, the depolymerization portion of the cycle occurs very slowly (i.e., over 3 days) by dialysis to remove salt, and should not be rushed. This protocol takes a total of 7 days and should yield 20 to 30 mg of labeled actin, which is good for >1 year when stored at  $-80^{\circ}\text{C}$ .

***Materials***

Muscle (rabbit psoas or chicken breast) acetone powder  
 1 M KCl (APPENDIX 2A)  
 1 M  $\text{MgCl}_2$  (APPENDIX 2A)  
 100 mM ATP (see recipe)  
 Succinimidyl ester derivative of the fluorescent probe of choice, pre-equilibrated to room temperature  
 Anhydrous DMSO

1 M sodium bicarbonate (see recipe), freshly prepared  
 1 M  $\text{NH}_4\text{Cl}$  (see recipe)  
 G-buffer, 4°C (see recipe; ATP should be added just prior to use)  
 50-ml beaker  
 Refrigerated ultracentrifuge with rotor (e.g., Beckman 70.1 Ti rotor)  
 13.5-ml screw-cap ultracentrifuge tubes  
 50-ml and 1-liter graduated cylinder  
 Spectrophotometer and quartz cuvette  
 1.6-ml microcentrifuge tubes  
 Aluminum foil  
 Refrigerated centrifuge with rotor (e.g., Sorvall SS-34 rotor)  
 50-ml open-top polycarbonate centrifuge tubes (or equivalent)  
 7-ml dounce homogenizer with type “B” pestle  
 Pretreated glycerol-free cellulose dialysis tubing (see recipe), ~2 cm diameter,  
 20,000 MWCO (i.e., Spectra/Por 6, Fisher)  
 Dialysis tubing clips  
 0.6-ml UV-impermeant microcentrifuge tubes

### ***Extract and polymerize actin monomers from muscle***

1. Add 1 g of muscle acetone powder to 25 ml chilled deionized, distilled water in a 50-ml beaker. Extract the actin monomers by mixing 30 min at 4°C. During extraction, cool the ultracentrifuge and rotor to 4°C.

*Use a magnetic stirrer and stir plate in the cold room.*

*Muscle acetone powder is commercially available from Sigma, but actin preparations from commercial sources will not give the best yields. A better source is from a laboratory that purifies actin regularly (generally, these laboratories store large frozen quantities) or to prepare the powder oneself using the method of Pardee and Spudich (1982).*

2. Transfer to 13.5-ml ultracentrifuge tubes and clarify the extract by ultracentrifuging 1 hr at  $185,000 \times g$  (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.

*Be sure to balance paired tubes by weight to within 0.01 g.*

3. After the centrifugation, carefully collect supernatant in a 50-ml graduated cylinder. Determine the initial amount of actin monomers by measuring the absorbance at 290 nm ( $A_{290}$ ) of a 1:10 dilution and using the extinction coefficient of actin  $\epsilon = 0.62 \text{ M}^{-1} \text{ cm}^{-1}$  in the equation for Beer's law (see Support Protocol 1, step 35).
4. Polymerize the actin by adding 1 M KCl (final 0.1 M), 1 M  $\text{MgCl}_2$  (final 2 mM), and 100 mM ATP (final 1 mM). Transfer to a 50-ml beaker and stir 30 min at room temperature.
5. During the polymerization, dissolve the fluorophore in anhydrous DMSO to a concentration of 100 mM in a 1.6-ml microcentrifuge tube. Warm fluorescent dye solution for 10 min to 37°C and vortex vigorously.
6. Clarify the dye solution by microcentrifuging 5 min at maximum speed, room temperature. Promptly transfer the supernatant to a fresh 1.6-ml microcentrifuge tube and keep at room temperature.
7. After polymerization, raise the pH of the actin solution to ~pH 9 for the labeling reaction by adding 1/5 vol of 1 M sodium bicarbonate.

### ***Label actin***

8. Add fluorescent dye solution to the protein solution at a ratio of 5 mole fluorophore to 1 mole actin. Add a stir bar to the beaker, cover the beaker completely with aluminum foil, and stir 45 min at room temperature.
9. After 45 min, add a second aliquot of fluorophore, equal to the amount of the first addition, re-cover the beaker, and continue stirring for an additional 45 min, room temperature.

*During this time, chill the super-speed centrifuge and rotor to 4°C.*

10. Following labeling, quench the reaction by adding 1 M  $\text{NH}_4\text{Cl}$  to a final concentration of 50 mM. Stir 10 min, 4°C.
11. Transfer the solution to 50-ml super-speed centrifuge tubes and pellet large aggregated material by centrifuging 10 min at  $12,000 \times g$  (Sorvall SS-34 rotor at 10,000 rpm), 4°C.
12. Collect the supernatant and transfer to new 50-ml screw-cap ultracentrifuge tubes. Balance the tubes to within 0.01 g and ultracentrifuge the F-actin 60 min at  $185,000 \times g$  (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.
13. Note the position of the pellet in the ultracentrifuge tube and aspirate the supernatant. Resuspend pellet in 5 ml of G-buffer.
14. Pool pellets in a 7-ml dounce homogenizer and homogenize slowly on ice to resuspend, being careful not to introduce bubbles; then thoroughly rinse the ultracentrifuge tubes with an additional 1 ml of G-buffer.

### ***Depolymerize the F-actin***

15. Transfer the resuspended pellets into pretreated dialysis tubing (clipped at one end to form a bag) that has been rinsed with G-buffer. Squeeze out the bubbles and seal the opposite end with another clip. Place the dialysis bag in a 1-liter graduated cylinder containing 1 liter G-buffer and a magnetic stir bar. Cover the entire cylinder with aluminum foil, and stir for 3 days in a cold room, changing the buffer once every day.
16. Collect the dialyzed actin, transfer it to 13.5-ml ultracentrifuge tubes, and clarify the actin solution by ultracentrifuging 1 hr at  $185,000 \times g$  (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.
17. Collect the supernatant containing G-actin into a small beaker and discard the pellet.

### ***Polymerize G-actin to F-actin***

18. Polymerize G-actin into F-actin by addition of 1 M KCl (final 0.1 M), 1 M  $\text{MgCl}_2$  (final 2 mM), and 100 mM ATP (final 1 mM); and mix 60 min at 4°C.
19. Transfer the solution to an ultracentrifuge tube and pellet the F-actin by ultracentrifuging 60 min at  $185,000 \times g$  (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.
20. Aspirate the supernatant and resuspend the pellet in 1 to 2 ml of cold G-buffer in the dounce homogenizer. Transfer to a dialysis bag.
21. Dialyze the resuspended pellet in 1 liter G-buffer in a graduated cylinder covered with aluminum foil for 3 days, 4°C, changing the buffer once every day.

*Add ATP fresh to each change of dialysis buffer*

22. Following dialysis, transfer the dialysate to an 13.5-ml ultracentrifuge tubes and clarify the G-actin by ultracentrifuging 1 hr at  $185,000 \times g$  (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.

#### **Analyze actin**

23. Collect the supernatant and determine the concentration, total protein, percent yield, and fluorophore-to-actin ratio of the final product (see Support Protocol 1, step 35 for tubulin labeling).

*The extinction coefficient for actin at 290 nm is  $0.62 M^{-1} cm^{-1}$ , the molecular mass of actin monomer is 45 kDa, and the extinction coefficient for the fluorophore is supplied by the manufacturer.*

24. Drop-freeze the labeled actin in 10- $\mu$ l aliquots in 0.6-ml UV-impermeant microcentrifuge tubes and store at  $-80^{\circ}C$ .
25. For use for microinjection, rapidly thaw an aliquot of concentrated labeled actin and transfer immediately to ice. Dilute to 0.5 mg/ml with cold IB, and drop freeze 3- $\mu$ l aliquots for use within 2 weeks.

### **REAGENTS AND SOLUTIONS**

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.*

#### **Ammonium chloride ( $NH_4Cl$ ), 1 M**

5.35 g  $NH_4Cl$   
100 ml distilled  $H_2O$   
Store up to 6 months at room temperature

#### **ATP, 100 mM**

8.5 ml  $H_2O$   
0.55 g  $Na_2ATP$   
pH to  $\sim 7.0$  with NaOH  
Bring to final volume to 10 ml with  $H_2O$   
Dispense into 200- $\mu$ l aliquots  
Store up to 1 year at  $-20^{\circ}C$

#### **BRB-80, 10 $\times$**

400 mM K PIPES, pH 6.8 (see recipe)  
5 mM  $MgCl_2$  (APPENDIX 2A for 1 M)  
5 mM EGTA (UNIT 13.1)  
Store up to 1 year at  $4^{\circ}C$

#### **Buffered filming medium**

14.8 g/liter Leibovitz L-15 medium powder lacking phenol red (Sigma)  
7 mM HEPES, sodium salt  
1 $\times$  antibiotic/antimycotic (100 $\times$ , Life Technologies; penicillin/streptomycin/amphotericin B)  
10% (w/v) fetal bovine serum (FBS, APPENDIX 2A)  
Adjust pH to 7.2 and sterile filter  
Store up to 3 months at  $4^{\circ}C$

***CB/BRB-80 conversion buffer***

1.18 M K PIPES, pH 6.8 (see recipe)  
11 mM MgCl<sub>2</sub> (APPENDIX 2A for 1 M)  
Store up to 6 months at room temperature

***G-buffer***

2 mM Tris-Cl, pH 8.0 (APPENDIX 2A)  
0.2 mM CaCl<sub>2</sub> (APPENDIX 2A)  
Add just before use:  
0.2 mM ATP (see recipe for 100 mM)  
0.5 mM 2-mercaptoethanol  
Store up to 1 day at 4°C

***Glycerol-free cellulose dialysis tubing, pretreated***

Boil 10 min in 10 mM EDTA. Store in sealed jar for up to 1 year at 4°C. Rinse well with water before use.

***GTP, 100 mM***

Check molecular weight of GTP lot to determine the amount required for 10 ml of a 100 mM solution  
8.5 ml distilled H<sub>2</sub>O  
1 ml of 1 M MgSO<sub>4</sub> (APPENDIX 2A)  
Adjust pH to 7.0  
Distilled H<sub>2</sub>O to 10 ml  
Dispense into 200-μl aliquots, store up to 1 year at -20°C

***HEPES, 1 M (pH 8.6)***

119.15 g HEPES (free acid)  
Distilled H<sub>2</sub>O to 400 ml  
Add solid KOH a few pellets at a time while mixing until the pH is ~8.4  
Add concentrated KOH dropwise to achieve pH 8.6  
Distilled H<sub>2</sub>O to 500 ml  
Sterile filter and store up to 1 year at 4°C

***High-pH cushion***

0.1 M HEPES, sodium salt, pH 8.6 (add from 1 M stock; see recipe)  
1 mM MgCl<sub>2</sub> (APPENDIX 2A)  
1 mM EGTA (UNIT 13.1)  
60% (w/v) glycerol  
Store up to 6 months at room temperature

***Injection buffer (IB)***

50 mM potassium glutamate  
0.5 M MgCl<sub>2</sub> (APPENDIX 2A)  
Store up to 2 years at -20°C

***Labeling buffer***

0.1 M HEPES, sodium salt, pH 8.6  
1 mM MgCl<sub>2</sub> (APPENDIX 2A)  
1 mM EGTA (UNIT 13.1)  
40% (w/v) glycerol  
Store up to 6 months at room temperature

***Low-pH cushion***

1× BRB-80 (see recipe for 10×)  
60% (w/v) glycerol  
Store up to 6 months at room temperature

### ***Microinjection needles***

For Eppendorf systems, standard femtotip microinjection needles work well. For home-pulled needles, a blunt-tipped needle with a ~0.5- to 1.0- $\mu\text{m}$  i.d. tip opening should be used. Either needle type should be pre-coated inside and out with hexamethyldisilazane (HMDS, Pierce Chemical). This is done by propping the needles on a piece of modeling clay in a petri dish, putting a couple of drops of HMDS on the bottom of the dish, quickly covering the top, and putting the dish in the fume hood overnight to allow the HMDS to vaporize and coat the needles. This treatment greatly reduces needle-clogging problems. Needles may be tested by watching flow of a 1 mg/ml fluorescent dextran solution from a needle immersed in culture medium using a 40 $\times$  lens and epifluorescent illumination. At 1- to 2-psi needle pressure, the solution should flow *very* slowly from the needle, i.e., a small “cloud” of fluorescence as big as ~1/4 the field of view should take 2 to 4 sec to form.

### ***PIPES, 1.5 M (pH 6.8)***

226.8 g PIPES (free acid)

H<sub>2</sub>O to 400 ml

Add solid KOH a few pellets at a time while mixing until the pH is ~6.6

Add concentrated KOH dropwise to achieve pH 6.8

Distilled H<sub>2</sub>O to 500 ml

Sterile filter and store up to 1 year at 4°C

### ***Quench***

2 $\times$  BRB-80 (see recipe for 10 $\times$ )

100 mM potassium glutamate

40% (w/v) glycerol

Store up to 6 months at room temperature

### ***Sodium bicarbonate, 1 M***

8.40 g NaHCO<sub>3</sub>

100 ml distilled H<sub>2</sub>O

Make up fresh and keep at room temperature

## **COMMENTARY**

### **Background Information**

#### ***Principles of FSM image formation***

FSM was originally characterized for imaging fluorescent microtubules, thus it is important to understand the basics of microtubule assembly and structure in order to understand FSM images of microtubules. Similar principles apply to FSM images of actin.

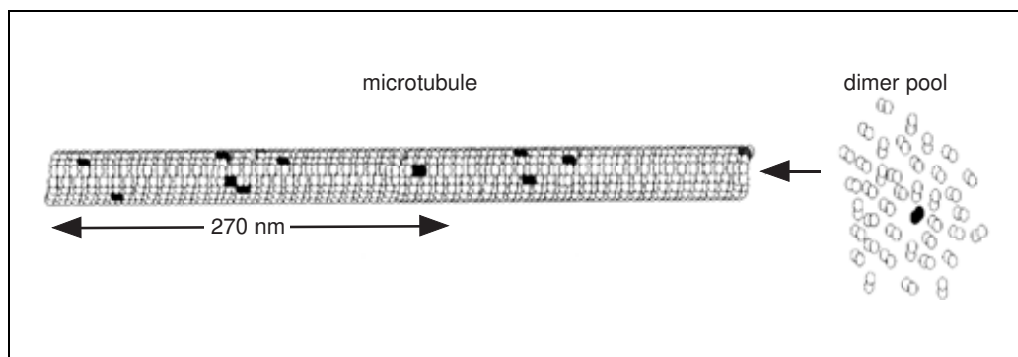
Microtubules dynamically assemble in cells from a cytoplasmic pool of  $\alpha/\beta$  tubulin dimers (reviewed by Desai and Mitchison, 1997). Dimers of 8  $\times$  5-nm are oriented head-to-tail along the 13 protofilaments that comprise the 25-nm diameter cylindrical wall of a microtubule (Fig. 4.10.4). There are 1625 dimers in 1  $\mu\text{m}$  of microtubule length. Microtubules grow by dimer association with their ends. Thus, microtubules can be fluorescently labeled by attachment of chemical fluorophores to purified

tubulin dimers, then incorporation of the fluorescent dimers into the ends of a microtubule polymer. As seen in Figure 4.10.1, microtubules assembled from a low fraction (~0.5%) of fluorescent tubulin appear speckled in high-resolution images, while microtubules assembled from higher fractions (~10%) of labeled tubulin appear continuously labeled along their lengths.

The resolution of fluorescent microtubules in the light microscope depends on the emission wavelength of the fluorophore (Inoué and Spring, 1997). Resolution limit in wide-field fluorescence microscopy is given by

$$r = 0.61\lambda/\text{NA}_{\text{obj}}$$

where  $\lambda$  is the wavelength of emission light and  $\text{NA}_{\text{obj}}$  is the numerical aperture of the objective lens. In practice, this equation means that the image of a single fluorophore will be the same



**Figure 4.10.4** Stochastic growth model for how microtubules get fluorescent speckles. See Background Information.  $R$  (red fluorescence) =  $0.61\lambda/NA_{\text{obj}} = 270 \text{ nm} = 440$  tubulin dimers;  $M = FN = 4.4$  for 1% labeled tubulin;  $SD = [Nf(1-f)]^{0.5} = 2.15$ ;  $C = SD/M = 0.48$ ; as mean,  $M$ , decreases, standard deviation,  $SD$ , increases, and speckle contrast,  $C$ , increases.

size as the image of however many fluorophores that can fit in a resolution-limited spot, only the single fluorophore will be much dimmer than the group. For example, resolution is  $\sim 270 \text{ nm}$  for the 620-nm fluorescence from tubulins conjugated to the X-rhodamine fluorophore. Thus, the image of a linear, 25-nm wide, red fluorescent microtubule is a linear series of resolution-limited 270-nm-wide fluorescent spots along the microtubule, with the intensity of the resolution-limited spots related to the number of fluorophores within each spot.

Analysis of the assembly of purified tubulins in vitro (Waterman-Storer and Salmon, 1998) has shown that fluorescent speckles in microtubule FSM images are generated by the normal stochastic association of fluorescently labeled and unlabeled tubulin dimers with growing microtubule ends (Fig. 4.10.4). Each time a dimer is added to the microtubule end, the probability that it will be a dimer with a fluorophore depends on the fraction ( $f$ ) of labeled dimers in the tubulin pool. If, for example,  $f = 1\%$ , then each time a dimer is added to a microtubule end, there is a 1 in 100 chance it will have bound fluorophore. Over many microns of microtubule length ( $1625 \text{ dimers}/\mu\text{m}$ ), the mean number of fluorescent dimers ( $M$ ), in 270 nm, the size of the resolution-limited spot, is:

$$M = fN$$

where  $f$  is the fraction of labeled fluorophores and  $N$  is the number of dimers in 270 nm of microtubule, or 440 dimers. For a situation in which  $f = 1\%$ , the average number of fluorescent dimer per 270 nm is  $M = 4.4$ . In FSM images, the speckle pattern along the microtubule is produced by variations from the mean in the number of fluorophores per resolution-

limited spot. The standard deviation ( $SD$ ) for a stochastic process is

$$SD = (Nf(1-f))^{0.5}$$

which is approximately the square root of the mean for small values of the fraction of labeled tubulin. For a mean value of 4.4,  $SD = 2.1$ . This high standard deviation relative to the mean explains how microtubules get fluorescent speckles as a result of a high variability in the number of fluorescent tubulin subunits per resolvable unit distance along the microtubule. The author defines speckle contrast ( $C$ ) as:

$$C = SD/M$$

At low fractions of fluorescent dimer, the standard deviation is high relative to the mean, producing more contrasting speckles. For the microtubule FSM image seen in Figure 4.10.1, the fraction of labeled tubulin of  $\sim 0.5\%$  or less is estimated. At this concentration, the number of fluorophores per diffraction-limited spot has been calculated to be between 0 and 7 (Waterman-Storer and Salmon, 1999).

The above analysis also does not consider the effects of background fluorescence on speckle contrast. As the fraction,  $f$ , of fluorescently labeled tubulin decreases, background fluorescence also decreases, giving less out-of-focus fluorescence and higher speckle contrast in the FSM images.

## Critical Parameters and Troubleshooting

The critical parameters for setting up a time-lapse FSM imaging system will not be discussed here as Basic Protocol 1 dealt with this in detail. Similarly, troubleshooting hardware and software problems is virtually impossible without knowledge of the specific components



of the imaging system. These problems are best dealt with by consultation with the manufacturer as problems arise.

For successful live-cell FSM imaging, the most critical components are proper fluorescent protein concentration, successful suppression of photobleaching, and high quality, well labeled, fully functional protein. Fluorescent speckles cannot be detected if the concentration of fluorescent protein in the cell is too high. Although, the needle concentrations recommended have been used successfully, the amount of protein that gets incorporated into the cell depends on the microinjection technique. Tubulin can be diluted with IB and actin can be diluted with G-buffer to alleviate problems of overly bright cells. Conversely, if there is not enough labeled protein, the camera exposures required to capture an image will be excessively long and may result in motion artifacts in the image. This can easily be solved by increasing the needle concentration.

Photobleaching problems can occur if the imaging chamber is not fully sealed, if there are air bubbles present in the chamber, or if the potency of the Oxyrase is wearing out. Make sure the edges of the coverslip are dry when attaching it to the tape and use Oxyrase that is stored for <1 year at  $-80^{\circ}\text{C}$ . Labeled tubulin or actin that is incompetent for polymerization due to denaturation or blockage of protein-protein interaction sites by dye binding will not contribute to forming fluorescent speckles by incorporating into microtubules or actin filaments, but it will surely contribute to soluble background fluorescence, making the dimly fluorescent speckled structure difficult to detect above the background. If the background is too high and the protein is not incorporating into polymers, the labeling will have to be carried out again.

The points to pay attention to during labeling of fluorescent tubulin to increase yield and produce well-labeled, functional protein is to not over-label the protein by extending the labeling reaction time, to save time by having solutions and centrifuges pre-equilibrated to the proper temperatures, and to be meticulous in resuspending pellets and collecting supernatants. After the first microtubule polymerization and centrifugation, when resuspending the microtubules for labeling, keeping the pellets warm and using warm buffer for resuspension is essential. This is a step where much tubulin can be lost if it is allowed to depolymerize prior to labeling. After centrifuging the labeled microtubules, resuspension of the labeled micro-

tubule pellet and depolymerization is another step where yield can be reduced. If the pellet is not completely resuspended, the microtubules will not depolymerize and will be lost in the subsequent clarification step—thus, be patient and homogenize slowly during the entire depolymerization incubation. A final key point to note is that small amounts (<15 mg) of starting material when carrying out these protocols should not be used. Yield for the protocols is knowingly very low, and if small amounts are used to start with, one may end with no labeled product.

Actin labeling is quite easy because one begins with such a large amount of material and actin is such a stable protein that it is difficult to be unsuccessful. One key parameter is the quality of the acetone powder. Actin will not be readily extracted from poor powder. Use the most finely powdered or “fluffiest” powder within the supply. Do not include hard chunks or dark-colored particles in the starting material for the prep as these will not have been fully acetone extracted. It is not recommended to speed the depolymerization steps by decreasing dialysis time. This will cause large losses of protein, which will fail to depolymerize and subsequently be lost in the pellet of the clarification centrifugation. One may change dialysis buffers more often, but do not decrease dialysis time. In fact, one may increase the dialysis for up to 5 days if necessary. When changing dialysis buffers, always add the ATP fresh to the buffer.

The labeling efficiency of tubulin or actin should not have dramatic effects on imaging. However if a very poor labeling is achieved (<0.5 dye per protein), the large amount of protein microinjected to get good images may have some effects on the balance of polymer/monomer in the cell.

## Anticipated Results

When live cells are injected with low levels of well-prepared fluorescently labeled cytoskeletal proteins and imaged on an efficient epifluorescent microscope at high resolution with a sensitive, low-noise cooled CCD that is matched in resolution to the microscope optics, FSM images like those in Figure 4.10.1 should be obtained. In time lapse, microtubule FSM should reveal the assembly dynamics of speckled microtubule ends at the cell periphery as microtubules undergo dynamic instability, growing at 1 to 10  $\mu\text{m}/\text{min}$  and shortening at 5 to 20  $\mu\text{m}/\text{min}$ , with frequent switching between these states. The speckles on microtubules

should remain constant in pattern and only change after depolymerization and repolymerization. In this regard, keep in mind that very slight shifts in focus can change the intensity of speckles dramatically. For time-lapse actin FSM, actin speckles should appear along the leading edges of migrating cells and move towards the cell center at 0.1 to 2  $\mu\text{m}/\text{min}$ . In addition, within the lamella actin network, fluorescent speckles should appear and disappear as actin within the meshwork turns over. Actin in stress fibers and bundles will appear as linear arrays of speckles, and contractility can be seen as speckles move within these bundles.

### Time Considerations

Relative to preparing fluorescent proteins, performing time-lapse FSM of living cells requires little time and is dependent on the biological process of interest. The cells will need to be microinjected, to recover for ~1 hr after microinjection, to be mounted for live-cell microscopy, and then to be imaged on the microscope for as long as is required by the biological process of interest. Fluorescent labeling of tubulin requires 1 full day, provided tubulin has already been purified according to UNIT 13.1, Support Protocol 3. Fluorescent labeling of actin takes 7 days with the first day, in which the labeling is carried out, being the most labor intensive, with ~6 hr required. Day 2 and 3 need 10 min each, 4 hr on day 4, 10 min on both days 5 and 6, and 2 to 3 hr on day 7. The method should yield 20 to 30 mg of labeled actin. Both of these fluorescent proteins are good stored at  $-80^{\circ}\text{C}$  for a reasonably long time, although labeled actin appears to be more stable (>1 year) than tubulin (6 to 8 months).

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