**Video Article**

# Live Cell Imaging of F-actin Dynamics via Fluorescent Speckle Microscopy (FSM)

James Lim, Gaudenz Danuser
Department of Cell Biology, Scripps Institute

Correspondence to: James Lim at jilim@scripps.edu, Gaudenz Danuser at gdanuser@scripps.edu

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## Abstract

In this protocol we describe the use of Fluorescent Speckle Microscopy (FSM) to capture high-resolution images of actin dynamics in PtK1 cells. A unique advantage of FSM is its ability to capture the movement and turnover kinetics (assembly/disassembly) of the F-actin network within living cells. This technique is particularly useful in deriving quantitative measurements of F-actin dynamics when paired with computer vision software (qFSM). We describe the selection, microinjection and visualization of fluorescent actin probes in living cells. Importantly, similar procedures are applicable to visualizing other macomolecular assemblies. FSM has been demonstrated for microtubules, intermediate filaments, and adhesion complexes.

## Protocol

### Section 1: Obtaining your fluorescently labeled actin for FSM

Materials required: purified actin, fluorophore (Alexa, X-rhodamine recommended). G-buffer, ultracentrifuge

1. A key component to acquiring good FSM movies requires the proper labeling of actin (or other cytoskeletal proteins) with the fluorophore of your choice.
   We recommend using fluorophores of Alexa (488, 568 wavelengths) and X-rhodamine succinimidyl ester derivatives that target exposed lysine residues on the surface of actin.
2. When choosing your fluorophore and its appropriate wavelength, ensure that your microscope setup has the appropriate filters (excitation and emission) and illuminators (mercury arc lamp and/or lasers) to accommodate your fluorescent tag. We recommend selecting wavelengths in the orange to red wavelengths (560-630 nm) to minimize the effects of auto-fluorescence and photo-damage and to ensure its compatibility with GFP-conjugates.
3. Pure actin can be extracted from muscle tissue belonging to Rat or Chicken, but requires a lengthy extraction procedure from muscle acetone powder. Muscle acetone powder can be purchased from Invitrogen. The labeling procedure is not discussed in this protocol, but can be found here[1]
4. Alternatively, you can purchase labeled actin proteins from several vendors including Cytoskeleton Inc., and Invitrogen. Custom labeling services are also available through Invitrogen. Purified, non-labeled actin (both muscle and nonmuscle actin) can be purchased from Cytoskeleton Inc.
5. Whether you decide to purchase or prepare your own labeled actin, you want to ensure that the labeling ratio is somewhere around 0.3 to 0.7 dyes per monomer of actin. Too high a labeling ratio may effect the turnover kinetics (and binding to associated proteins) of the actin filament, while too low a labeling ratio will lead to poor looking speckles (low signal to noise) and may cause microinjection problems (see below). This is a critical factor for getting good speckle images.
6. Prior to long term storage, the labeled actin solution should be clarified by spinning at 75,000 - 80,000xg for 20 minutes at 4 degrees Celcius. Labeled actin should be stored in G-buffer solution and kept -80 degrees Celcius. Preparing 2 uL aliquots is highly recommended to reduce the detrimental effects of repeated freezing-thawing (leads to insoluble aggregates). Refrain from exposing the labeled actin solution to direct light (will photobleaching of fluorophores). We recommend using opaque microcentrifuge tubes for long term storage.

### Section 2: Preparation of Cells and Microinjection of labeled actin

Materials required: microinjection needles, microsyringe, microloaders, prewarmed cell media, injection buffer, bucket of ice, microscope (phase ring, phase contrast objective 40X, transjector, needle puller, micromanipulator)

1. Many cell types are amenable to FSM, but getting good speckle images is directly dependent on the success of the microinjection procedure. When selecting cells for FSM, cells should be large ( > 50 um in diameter), should spread when plated, and be naturally adherent to substrates (or plastic tissue culture dishes). It also helps if the cell's cytosol occupies an area greater than its nucleus. The aforementioned criteria ensure that cells are amenable to microinjection. Successful candidate cell lines include (but not limited to) PtK1[2,3], newt lung cells[4], and Aplysia neurons[3].
2. Cells should be plated on square glass coverslips (22 x 22 mm; no. 1-1/2) that have been acid-washed. Standard substrate coating can be applied to the coverslip and will not affect microinjection.
3. The correct selection of microinjection needles is dependent on the size of the tip opening. Typically, hole sizes can very in size from 0.5 um to as large as 3 um. However, we recommend that injection of actin requires an optimal opening size between 0.5 to 1 um. If clogging problems are encountered consistently, larger hole sizes can accommodate for the delivery of monomeric as well as unwanted polymerized actin filaments. Microinjection needles can be purchased through several vendors including Eppendorf systems which make femtotip microinjection needles. Alternatively if a commercial needle puller is available, glass microinjection tubes can purchased from Sutter, and pulled to specification. For beginners, we recommend having 10-20 pulled needles on hand before starting the injection procedure.
4. Ideal 'injection concentration' (final concentration of actin loaded in your needle) of actin is between 0.5 to 1.0 ug/ul based on a 40% labeling ratio of dye to actin monomer. Lower labeling ratio of your actin can be compensated by injecting at higher concentrations (1-3 ug/ul).

 

However, the clogging frequency of your needle will be increased. This can be partially alleviated by using needles with larger openings. Stock concentration of actin can be diluted by adding ice-cold injection buffer. Ice-cold ddH2O can also be used to dilute for quick injections. At this point all working solutions should be kept on ice.

5. Load 0.5 to 1.0 uL of your actin solution using a microsyringe (narrow-gauge – Hamilton), or alternatively by using a microloader pipette tip.
6. Carefully dispense the actin solution, by gently releasing the solution. Bubbles should be avoided, but can be easily removed by taking up excess liquid surrounding the bubble.
7. Ensure that the actin solution is resting completely at the needle tip. Excess solution anywhere along the capillary of the needle can disrupt the injection flow.
8. Now, carefully attach the needle, avoiding contact with the needle tip, to the injector holder. Position the holder so that the needle makes a 35-50 degree angle to the base of the microscope stage. Lower angles are preferred.
9. Lower the tip of the needle until it barely breaks the surface of the media bathing your cells.
10. Now position the needle tip (without lowering) so that it rests directly above the center of the objective. If the needle tip is aligned with the objective, you should see a bright halo through the eyepiece. This is the end of the needle tip. Slowly moving the needle side to side (using the manipulator) may help better locate the halo.
11. Adjust the z-focus on the microscope until your cells are in clear view. Now, slowly lower the tip until the halo gradually constricts until finally converging into the needle tip – you will need to control the focus (z-position) simultaneously. An additional feature to look for is the shaft of the needle, which will cast linear shadows. Lowering the needle will gradually define the needle shaft. When done correctly the end of the needle and your cells should both be in clear view. Slight adjustment of the phase ring may be required to improve contrast.
12. When navigating, ensure the needle tip is well above your cells to prevent breaking the needle tip.
13. When selecting cells to inject, cells with free edges should be facing the needle tip. This will ensure declining slope of the cell (from thickest point, where the nucleus resides, to the thinnest point the leading edge) will meet the needle tip, rather than running parallel to it.
14. The needle pressure should be set at 0.3 to 0.8 psi. The constant pressure applied will result in a steady flow of the actin solution.
15. After deciding on a cell to inject, position your needle so the tip is next to the nucleus (perinuclear region, thickest part of the cell where the nucleus is not directly below).
16. Slowly lower, and readjust the position of the needle until the tip is depressing the membrane, now incrementally lower until the needle tip has pierced the membrane. If the needle has fully pierced the membrane, the cell will appear brighter, as soon as you see this difference raise the needle until it is no longer touching the cell. The actual piercing and injecting process will take 0.5 to 2 seconds.
17. When done correctly, the cell shape will appear unchanged, whereas too much injection will result in quick retraction of the cell edge and in some cases you will actually see the cell explode.
18. If you do not see changes in the cell when microinjecting (does not get brighter), the needle tip may be closed. You can test this by pressing the 'clean' button if available on your transjector. If open, this will significantly increase the constant pressure flow which can be seen through the eyepieces – it will appear as ripples and nearby debris can be seen dispersing.
19. If the needle tip is closed, you can choose to insert a new microinjection needle or attempt to break the tip of the needle by gently lowering the needle until the tip of the needle presses against the glass coverslip, thus causing the very end of the tip to break. The breakage area should be no more than a $1/5^{th}$ of the area of the needle point (the portion of the needle that starts to converge to a point).
20. Continue with the injection, spending no more than 20 – 45 minutes for each microinjection session (will depend on cell type). As a general rule, shorter sessions are recommended to minimize stress on cells.
21. After finishing the microinjection, change the cell media in the dish by adding fresh prewarmed media. Then allow cells to recover for 30 minutes in an incubator before imaging. This will also allow for the actin to integrate into the existing cytoskeleton network.

## Section 3: Assembling the Imaging Chamber

Materials Required: coverglass, double sided tape, q-tips, forceps, valap, kim wipe, oxyrase, imaging media, razor blade, p200 pipette, pure ethanol

1. Begin by prewarming the imaging media and thawing a 15 uL aliquot of Oxyrase. Make sure to protect the photosensitive oxyrase from light. The addition of oxyrase will greatly reduce the effects of photobleaching.
2. Prewarm the valap, by setting the hot plate temperature to low. Do not overheat/burn the valap for it will lose its efficacy as a sealant.
3. Wipe down a coverglass with a kimwipe doused with pure ethanol to clean and remove small debris.
4. Stack two equal sized lengths (2.5 cm long) of double-sided tape on top of each other, using a clean flat surface as a platform. Press out any trapped air bubbles to create a completely flat plane.
5. Using a razor blade, cut two straight strips along the long axis, so that each strip measures 0.5 cm in width.
6. Using forceps, place each strip against the long edge of your coverglass so that the two strips of tape creates a gap in the center of your coverglass. Press gently on the tape to make a nice seal with the glass. The strips of tape will act as spacers between the coverglass and the coverslip.
7. Make sure the valap has completely liquefied before you begin the next step.
8. Retrieve your prewarmed imaging media, adding 15 ul of oxyrase to every 500 uL of media.
9. Fold kimwipes into triangles to create 3 hard edges. This will be used to create a dry surface for the tape to stick on.
10. Retrieve your cells from the incubator. Using forceps, grab a corner of the coverslip. Quickly place the coverslip on a kimwipe (cell side is facing up, not touching the tissue), and use the folded kimwipe to wipe two opposing sides of the coverslip to create a semi-dry surface (on the surface where your cells rest).
11. Using forceps, grab a corner the coverslip and place it on your coverglass so that the dry surfaces are aligned to the two strips of tape. Notice now you have two closed sides and two open slits. Slowly pipette 200 uL of your imaging-media-oxyrase solution close to one opening. Importantly, no air bubbles should be introduced. The solution should be sucked up into the newly assembled chamber by capillary forces, thus fully immersing your cells in solution.
12. Using a Q-tip dab the four corners of your coverslip with melted valap to quickly affix and stabilize the coverslip. You'll notice that the valap dries instantly (within seconds at room temperature).
13. Now beginning with the open sides (non-tape side), lay a thin strip of valap by using a Q-tip and mimicking a brushing stroke. Repeat for the closed sides. Slowly build up the coat of valap, thus reinforcing the seal by repeating the coating steps. The valap should be confined to the edges, leaving the center of the coverslip clear.
14. Gently clean the center portion of the coverslip by using a Q-tip with ddH2O to remove any residual media, being mindful not to crush the cells. Repeat cleaning step with pure ethanol.
15. You will now have a fully enclosed imaging chamber optimized for speckle imaging. Having a fully sealed enclosure will prevent your fluorophores from quickly photobleaching.

 

## Section 4: Imaging cells

Materials required: inverted widefield microscope, mecury lamp, appropriate filters, cooled CCD camera with 6.7 micron pixels, High numerical aperture, oil-immersion plan-Apochromatic objectives (60X to 100X, phase or DIC). Vibration table. Imaging software.

1. Prewarm the microscope stage to 37 degrees. Place your newly completed imaging chamber, and wait 10-15 minutes for the temperature to stabilize before imaging.
2. Start by focusing your cells in Bright-field, and then find your injected cells by toggling the correct fluorescence settings. Try to minimize the time of fluorescence exposure to reduce photobleaching.
3. Look for injected cells that have healthy curved leading edges. Over-injected cells (exploded cells) will be characterized by retracted and jagged edges, resembling numerous filopodia – avoid imaging these cells.
4. Acquisition settings will depend on the cell type, the quality of the labeled actin, and microscope components. However, exposure settings should not be greater than 2 seconds, and time interval between frames should be set between 5 and 10 seconds. Typical lengths for a time series (per imaged cell) can vary anywhere between 10 to 30 minutes, and is limited by photobleaching effects. Gain settings on the camera can be turned up to increase the signal to noise.
5. Ideal speckle characteristics should have each speckle spaced by ~2 speckle diameters to the next neighboring speckle. This will warrant optimal spatial and temporal sampling of the actin structure. For epithelial cells, the speckled actin network will appear homogeneous, with speckles evenly spread apart. Cell lines that feature dense stress fibers or lamellipodia will be distinguishable, but will have a dotted/speckled appearance. Exposure settings should be adjusted to get the best speckled images.
6. Over-microinjected cells will have densely packed speckles that do not appear to be discrete. Stress fibers and the lamellipodia will lose its dotted appearance.
7. Under-microinjected cells will appear very dim through the eyepiece, containing speckles that are far apart (>10 speckles apart) and appearing randomly scattered. Stress fibers and the lamellipodia in these cells will be indistinguishable.
8. Key to obtaining ‘good’ speckle movies is maintaining focus. This is particularly important for post-analysis involving speckle flow and turnover measurements. Thus the quality of the quantitative analysis will depend on locking in the focus for the duration of the time-lapse. This can be particularly challenging if the imaging plane is quite thin. Other factors will depend on the stability of the microscope system, particularly the stage housing and the imaging chamber. Contrast-based focusing can be used if image interval lengths are sufficient to accommodate this feature. Other options include purchasing a near-infrared based focusing system that provides reliable real-time focus adjustments. You can find these offerings from Nikon, Olympus and ASI.

## Section 5: qFSM analysis

This section is not discussed in detail, but the information on the analysis software can be found here [5]. Software download requests can be made on our website (lccb.scripps.edu).
Topics Covered:

- Noise calibration
- Speckle detection
- Tracking using a hybrid approach of image correlation-based tracking of the coarse motion of speckle areas and single particle tracking of the detailed motion of individual speckles.
- Computation of the polymer assembly and disassembly rates from the intensity fluctuations during speckle appearance and disappearance events.

## Discussion

Several key factors are crucial for successfully obtaining speckle images, however good speckles begin and end with the quality of your fluorescently labeled actin. A high labeling ratio of dye to actin monomer, set around 0.4 to 0.7 will ensure that speckles will appear bright and discrete, while the labeled protein itself should be soluble and free of aggregates. Equally important is the microinjection procedure. To ensure normal cell homeostasis (and survival), cells need to be injected with a low flow pressure, introducing a low concentration of labeled protein. This requires some degree of technical expertise/experience with respect to the use of the microinjection system. As a general rule, shorter injection times (< 1s) are preferred over longer injection times. The last crucial component is the microscope. An inverted microscope paired with a mercury-lamp epi-illuminator, found in most core facilities, will serve as an adequate platform for speckle imaging. Assuming the proper fluorescence filters are in place, the combination of a high NA objective and a cooled CCD camera will produce high-resolution images of bright speckles. We recommend using high magnification (100X), high NA (>1.3), plan-apochromat objectives that will provide superior resolution and brightness. Under ideal injection conditions, the magnification can be lowered to 60X, increasing the brightness and SNR per pixel. Low noise, high quantum efficient, cooled CCD cameras, with pixel sizes of ~6.5 microns are ideal detectors, and in some cases can compensate for poor quality fluorescent probes. Additionally, fluorescently labeled protein probes can be co-injected with cDNA constructs (nucleoinjection) to express GFP/RFP conjugated proteins within the same cell. This requires the nucleoinjection of cDNA into the nuclei of cells, followed by a second injection in the surrounding cytoplasm. In summary, FSM provides unprecedented insights into cytoskeleton dynamics. Obtaining good speckles requires the proper probes, equipment and a little bit patience (training).

## Acknowledgements

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