Video Article

Labeling F-actin Barbed Ends with Rhodamine-actin in Permeabilized Neuronal Growth Cones

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Abstract

The motile tips of growing axons are called growth cones. Growth cones lead navigating axons through developing tissues by interacting with locally expressed molecular guidance cues that bind growth cone receptors and regulate the dynamics and organization of the growth cone cytoskeleton[3-6]. The main target of these navigational signals is the actin filament meshwork that fills the growth cone periphery and that drives growth cone motility through continual actin polymerization and dynamic remodeling[7]. Positive or attractive guidance cues induce growth cone turning by stimulating actin filament (F-actin) polymerization in the region of the growth cone periphery that is nearer the source of the attractant cue. This actin polymerization drives local growth cone protrusion, adhesion of the leading margin and axonal elongation toward the attractant.

Actin filament polymerization depends on the availability of sufficient actin monomer and on polymerization nuclei or actin filament barbed ends for the addition of monomer. Actin monomer is abundantly available in chick retinal and dorsal root ganglion (DRG) growth cones. Consequently, polymerization increases rapidly when free F-actin barbed ends become available for monomer addition. This occurs in chick DRG and retinal growth cones via the local activation of the F-actin severing protein actin depolymerizing factor (ADF/cofilin) in the growth cone region closer to an attractant[8-10]. This heightened ADF/cofilin activity severs actin filaments to create new F-actin barbed ends for polymerization. The following method demonstrates this mechanism. Total content of F-actin is visualized by staining with fluorescent phalloidin. F-actin barbed ends are visualized by the incorporation of rhodamine-actin within growth cones that are permeabilized with the procedure described in the following, which is adapted from previous studies of other motile cells[11, 12]. When rhodamine-actin is added at a concentration above the critical concentration for actin monomer addition to barbed ends, rhodamine-actin assembles onto free barbed ends. If the attractive cue is presented in a gradient, such as being released from a micropipette positioned to one side of a growth cone, the incorporation of rhodamine-actin onto F-actin barbed ends will be greater in the growth cone side toward the micropipette[10].

Growth cones are small and delicate cell structures. The procedures of permeabilization, rhodamine-actin incorporation, fixation and fluorescence visualization are all carefully done and can be conducted on the stage of an inverted microscope. These methods can be applied to studying local actin polymerization in migrating neurons, other primary tissue cells or cell lines.

Video Link

The video component of this article can be found at https://www.jove.com/video/2409/

Protocol

For rhodamine-actin labeling, neurons are cultured on glass coverslips placed in the bottom of 35 mm plastic dishes, or on coverslips glued into "video" dishes.

1. Preparation of Coverslips or "Video" Dishes

1. Many neuronal types are poorly adhesive to *in vitro* substrates. Glass coverslips, which are required for this procedure, may not allow sufficient neuron-substrate adhesion unless they are adequately cleaned and prepared. A detailed procedure for acid washing and cleaning glass coverslips is described in the book Culturing Nerve Cells, edited by Banker and Goslin. Alternatively, a nitrocellulose coating for coverslips, which avidly binds substrate molecules, is described in steps 1.7 and 1.8 below.
2. Glass coverslips (e.g. 18 mm squares or 24 mm circles) are rinsed in dH_2O and baked in dry heat (≥225°C) as long as possible (minimum of several days up to three months or longer) to remove traces of organic compounds. Go to step 1.6 or for video dishes proceed to 1.3.
3. To create a thin clear glass substrate for high resolution videomicroscopy, circular holes of appropriate diameter are drilled into the bottoms of 35 or 50 mm plastic Petri or tissue culture dishes using an electric cork borer (or similar instrument). Drilling is done slowly with light pressure to avoid cracking the plastic. Edges of the drilled hole are scraped with scissors to create a smooth surface on both sides.
4. Glass coverslips are cleaned for tissue culture use, as described in 1.1 or 1.2. Coverslips are glued in place over the holes, using non-toxic silicone aquarium cement (for 18x18mm coverslips, a 12mm diameter bit is used). The cement is cured for 24 hours.
5. (The following steps are done using sterile conditions) The video dishes are rinsed 3 times with sterile dH_2O and allowed to air dry.

6. Coverslips are coated with substrate molecules for neuronal culture. First, the coverslip surface is coated with (250 µL for 18x18 coverslip) a solution of 100 µg/ mL poly-D-lysine in PBS for 4-8 hrs (or overnight) in a humidified 38° C incubator. Coverslips are then rinsed 3 times with sterile dH_2O to remove unbound poly-D-lysine and allowed to air dry. Many neuronal types are cultured on coverslips coated with poly-D-lysine alone, if serum proteins are included in the culture medium. However, results more relevant to *in vivo* conditions are obtained by using natural substrate molecules. Solutions of these molecules, such as laminin, fibronectin or L1 CAM (1-20 µg/ mL in PBS), can be directly applied to poly-D-lysine coated coverslips for 4-8 hrs or overnight, but in our lab we routinely coat the prepared coverslips with a thin film of nitrocellulose before applying cell adhesion molecules to increase protein-substrate binding.
7. Make a 1% nitrocellulose solution by dissolving 5 g nitrocellulose in 5 mL of 100% amyl acetate. Apply 10 µL of this solution to the coverslip and gently spread it over the surface. Air dry 10 minutes.
8. Apply 250 µL of a solution of 25 µg/ mL laminin or 4 µg/ mL L1 CAM in PBS and spread over the nitrocellulose surface. Incubate 4 hours (or overnight) in a humidified 38° C incubator, aspirate substrate and immediately add culture medium, so the substrate does not dry.

2. Preparation of Neuronal Cultures

1. Embryonic day 7 chick embryos are removed from eggs, and placed in 100 mm petri dishes containing culture medium with 10% serum for dissection to remove the internal organs from the thorax and abdomen, using a stereomicroscope.
2. The embryos are then rinsed with medium and moved to a 60 mm dish with liquid medium and further dissected to remove and prepare explants of dorsal root ganglia (DRG) or neural retinal tissues. A detailed dissection description can be found in Methods in Cell Biology, volume 71, Neurons: Methods and Applications for the Cell Biologist, edited by Hollenbeck and Bamburg. DRG explants are 1/2-whole ganglia and neural retinal explants are 1 mm or less in diameter.
3. After cleaning the explants of extraneous tissues, individual explants are moved with watchmakers forceps to the coverslips, which are already in culture dishes with the culture medium. Under a dissecting microscope, the explant is gently pressed to the center of the coverslip with forceps to prevent movement while transporting to the incubator. Explants are cultured in F12 medium with B27 additives, buffered with 10 mM HEPES to pH 7.4 and placed overnight in a humidified 38° C incubator. Growth factors can be added, as needed, to the culture medium. Neurotrophins are often added to DRG cultures from older embryos, although E7 DRG neurons will extend axons from explants without added neurotrophins. Explants of neural retina extend axons in this culture medium without adding growth factors.
4. Cultures are incubated for at least 18 hours to allow sufficient axonal outgrowth before beginning the procedure below.

3. Preparation of Rhodamine-actin Permeabilization Buffer

1. A stock solution of permeabilization buffer containing 138 mM KCl, 10 mM PIPES, 3 mM EGTA, 4mM $MgCl_2$, and 1% BSA (pH = 6.9) can be kept up to 2 weeks at 4°C11. Just before use, the following components are added for a final concentration of: 0.025% saponin, 0.1 mM ATP, and 100 nM Alexa-fluor 350 phalloidin.
2. A separate solution is also freshly prepared just before use with these same components plus 0.45µM rhodamine non-muscle actin The solution is alternately vortexed and tritrated for 5 min to dissolve rhodamine-actin clumps, and is vortexed for 1 min during the initial permeabilization step to prevent polymerization in the tube. If despite this, filament assembly in the tube remains a problem, the solution can be centrifuged prior to use.

4. Permeabilization of Neuronal Cultures and Incorporation of Rhodamine-actin onto F-actin Barbed Ends

1. A culture dish with DRG or retinal explants is carefully removed from the incubator and the following steps are performed at room temperature.
2. All changes of solutions must be done carefully. The pipette tip should be placed at the edge of the coverslip, and solutions should be removed and added slowly and with minimal force.
3. The culture medium is removed by pipette carefully, but steadily, and enough permeabilization buffer to cover cells is added for 1 min (for 18mm x 18mm coverslip, ~60 µL). The culture is not rinsed with any other solutions before adding the permeabilization buffer, and the coverslip is not allowed to dry before adding the permeabilization buffer.
4. The permeabilization buffer is gently removed by pipette and replaced with the permeabilization buffer containing 0.45 µM rhodamine non-muscle actin for 4 min.
5. The rhodamine-actin-containing buffer is gently removed by pipette, and the neurons are fixed by adding a solution of 4% paraformaldehyde (laboratory grade, made with phosphate buffer, pH 7.3), 0.05% glutaraldehyde and 10% sucrose. After 5 minutes fixation the coverslips are gently rinsed with PBS, and mounted in Slowfade on 3-inch glass slides.
6. An alternative approach to changing solutions is to use a flow cell. This can be a commercially available flow cell, or a simple flow cell can be made by placing small pieces of plastic (≤ 1 mm thick) as spacers at the corners of the coverslip, and then mounting an equal-sized coverslip on top of the coverslip. Solutions can be exchanged by placing the pipette at one side of the flow cell and withdrawing solutions with a wick (cotton or a Kimwipe) at the other side.
7. The incorporation of rhodamine-actin onto F-actin barbed ends and fluorescent-phalloidin labeling of F-actin in growth cones is visualized with epi-fluorescence optics through a 60X oil immersion objective, and images are collected, using a cooled CCD camera. A confocal microscope may provide improved imaging.
8. For best quantification of rhodamine-actin incorporation all images should be collected in a single session, using the same gain and exposure settings of the digital camera sensitivity for each image. Analysis of labeling should be made from equivalent regions of each image; Metamorph, Image J or similar computerized tools for image analysis can be used.

VARIATIONS OF THIS PROCEDURE

5. Variation One: Collect Live Cell Images Prior to Rhodamine-actin Labeling

1. Video dishes are placed onto a warmed microscope stage, and live growth cone images are collected. Gridded coverslips can be used, or etchings and/or pen markings on the coverslip can be made to more easily find specific cells after labeling, or the video dish can be fixed in place on the microscope stage for the duration of the procedure.
2. Permeabilization, incorporation of rhodamine-actin, and cell fixation are conducted in the video dish, either on or off the microscope stage. After fixing, PBS is added to video dishes for immediate imaging. To preserve the sample for later imaging, slowfade mounting medium is added to the coverslip and a coverslip of equal size is gently placed on top (avoiding bubbles), and the edges are then sealed with clear nail polish.

6. Variation Two: Immunochemistry to Co-label Additional Proteins

1. Rhodamine-actin incorporation and fixation can be conducted on neurons cultured on coverslips or in video dishes, as described above (4.2-4.4).
2. After fixation, and rinsing in PBS, cells are treated 15 min with 0.1 M glycine in PBS and then extracted with 0.1% Triton X-100 (TX-100) in PBS with 2% goat serum and 1% BSA for 1 h. Coverslips are incubated with the primary antibodies diluted in PBS containing 1% BSA for 1 h. The coverslips are then rinsed and incubated in 0.1%TX-100 in PBS with 2% goat serum and 1% BSA for 1 h, before applying fluorescent secondary antibodies at 1:1000 dilution in PBS with 1% BSA for 1 h. After rinsing, the coverslips are again incubated in 0.1% TX-100 in PBS with 2% goat serum and 1% BSA for 30 min, rinsed, and mounted in anti-fading medium. The localization of some proteins is disrupted by the initial permeabilization step (4.3). To retain these proteins for localization with antibodies, 0.05% paraformaldehyde and 0.05% glutaraldehyde can be added to the permeabilization buffer for 1 min (i.e. before adding the rhodamine-actin) without affecting the barbed end labeling by rhodamine-actin.
3. Images of triple-labeled growth cones are collected by fluorescence or confocal microscopy.

7. Variation Three: Assessment of the Effects of Guidance Cues on F-actin Free Barbed Ends

1. A growth factor or guidance cue is added to culture dishes in the incubator at concentrations of several ng/ mL for a few minutes (or an appropriate treatment time) before removing the dish from the incubator and beginning the permeabilization procedure. This allows assessment of the short-term effects of growth factors or guidance cues on F-actin free barbed ends.
2. A video dish with neuronal cultures can be placed on a warmed microscope stage, and growth factors or guidance cues can be released from a micropipette in a gradient near a growth cone before beginning the permeabilization and rhodamine-actin incorporation. For example, NGF can be released for DRG neurons or netrin for neural retinal ganglion cells. The pipette can be introduced for as short as 1-2 minutes before beginning the permeabilization procedure. This allows assessment of the local effects of a guidance cue gradient on the formation of F-actin free barbed ends in growth cone leading margins (see reference citation 10 for images).
3. These variations can be combined with antibody-mediated labeling of other neuronal components, as described in 6.2 above.

8. Variation Four: F-actin Free Barbed End Labeling on Transfected Neurons

1. The use of constitutively active or dominant-negative constructs or protein knockdown using RNAi (with a fluorescent identifying marker) can be used to assess a protein's involvement in regulating F-actin free barbed ends. Depending on the protein, its localization within the growth cone, and if it is fused to a fluorescent tag, the fluorescent expressed protein in transfected cells may be lost upon permeabilization. If this is the case, video dishes can be used to identify a transfected cell microscopically before permeabilization, and the permeabilization can be performed on the microscope stage, after marking the positions of transfected cells. Alternatively, a GFP-actin construct can be co-transfected with a GFP-construct of interest. In this case, GFP-actin will be incorporated into F-actin, and will not be lost by permeabilization, enabling the identification of transfected cells after permeabilization.
2. This variation can be combined with the addition of guidance cues, as described in 7.1-7.3 above.

9. Representative Results

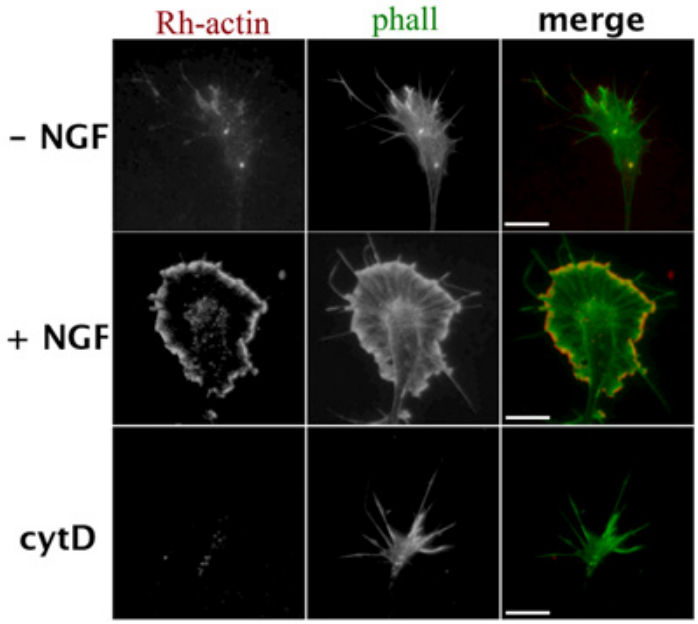

Figure 1. Global addition of NGF increases total F-actin and F-actin barbed ends at the growth cone leading margin. When a DRG growth cone is stimulated with nerve growth factor (NGF) for 5 minutes, actin polymerization is stimulated at the leading margin, and a bright band of rhodamine-actin labeling is seen around the growth cone periphery. Figure 1 compares rhodamine-actin incorporation into an unstimulated DRG growth cone and an NGF-stimulated DRG growth cone. The green fluorescence in the merged images is phalloidin labeling for F-actin in the growth cone and the red is rhodamine-actin labeling. A good control for the barbed end labeling is to add 10-6 M or higher cytochalasin B or D in the permeabilization buffer in steps 4.2 and 4.3. Cytochalasins cap F-actin barbed ends and inhibit rhodamine-actin binding to barbed ends. This should severely reduce or eliminate incorporation of rhodamine-actin into the cell. Scale bars, 10 μm.

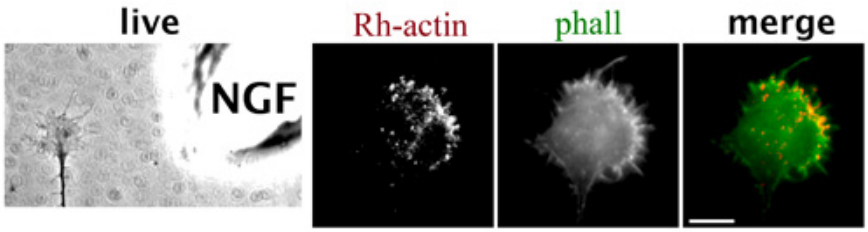

Figure 2. An NGF gradient locally increases F-actin barbed ends. A micropipette that releases NGF is brought to one side of a DRG growth cone for 2 minutes, followed by labeling of F-actin free barbed ends. The images below show that exposure to a gradient of NGF locally stimulates an increase in F-actin free barbed ends in the growth cone region closer to the pipette. The merged image shows phalloidin in green and rhodamine-actin in red. Scale bar, 10 μm.

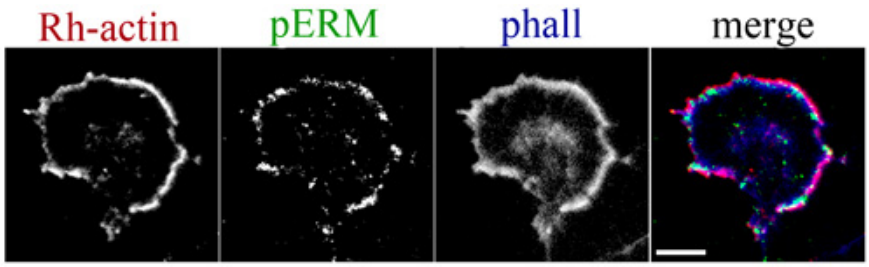

Figure 3. Activated ERM proteins accumulate at the growth cone leading margin. Immunocytochemical labeling of phospho-Ezrin/Radixin/Moesin (pERM) with F-actin free barbed ends. Gluteraldehyde (0.05%) and paraformaldehyde (0.05%) were added to the initial permeabilization buffer for one min to preserve ERM localization. Rhodamine-actin, red; pERM, green; phalloidin, blue. Scale bar, 10 μm.

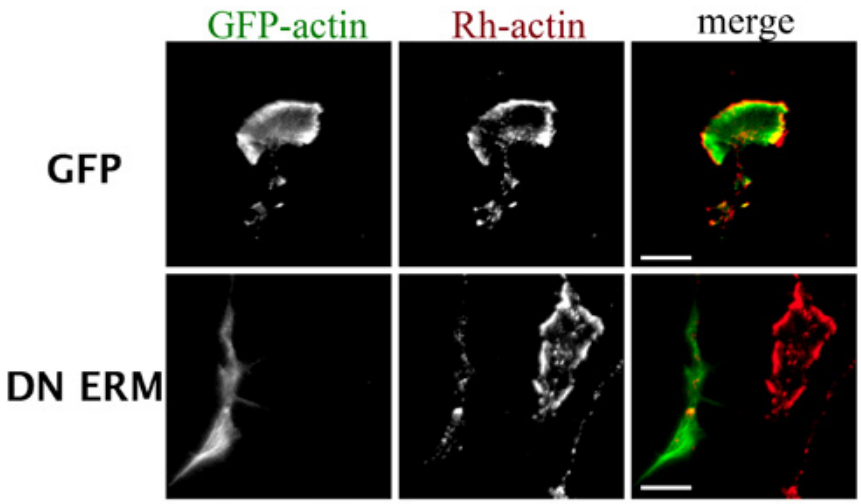

Figure 4. Stimulation of actin polymerization requires active ERM proteins. Dissociated DRGs were co-transfected with GFP-actin and a GFP control plasmid or a dominant-negative Ezrin/Radixin/Moesin (DN ERM) construct. The use of GFP-actin allows identification of transfected cells after permeabilization. Here, NGF was added 5 min prior to labeling of F-actin barbed ends. Note the growth cone transfected with DN ERM has reduced barbed end levels at the growth cone leading margin (lower middle panel), which contrasts with a nearby untransfected growth cone (lower panels), or with the GFP control (upper middle panel). Rhodamine-actin, red; GFP-actin, green. Scale bars, 10 μm.

Discussion

The methods presented here allow temporal and spatial resolution of cellular components that are involved in the dynamic remodeling of the actin cytoskeleton at the leading margin of migrating growth cones. The action of attractant molecules, like NGF or netrin, to rapidly stimulate actin filament polymerization is revealed as a local increase in actin barbed ends, created by actin filament severing by activated ADF/cofilin[10], as shown in Figures 1 and 2. The method permits localization of other proteins involved in mediating growth cone chemotropic responses, such as radixin, an ERM protein, pictured in Figures 3 and 4. These methods can also be applied to analyze the regulation of actin-based motility in migrating neurons, glial cells or other cell types.

The delicate nature of growth cones or other small motile structures is a significant limitation in the use of this method. Growth cone leading margins or similar motile regions of cells contain few structural elements other than actin filaments and the plasma membrane, so care must be taken at every step. Cell aggregates or explants may be disrupted by changes in surface tension, as solutions are exchanged. As mentioned in the protocol, it is best to place pipettes at the edge of coverslips for changing solutions, and the use of a flow cell would eliminate problems from surface tensions.

An alternative method to visualize actin barbed ends in motile regions of cells involves cell transfection to express fluorescent analogs of barbed end-binding proteins, such as Ena/VASP or myosin X. However, actin dynamics in growth cone margins may be changed by unregulated expression of these actin regulatory proteins.

Filopodia are not as strongly labeled by this rhodamine-actin labeling method as are lamellipodia. The filopodia may be disrupted, although they are labeled and stabilized by the fluorescent-phalloidin contained in the permeabilization buffer. This difference in lamellipodial vs filopodial incorporation of rhodamine-actin might reflect a quantitative limitation in the visualization of the incorporated rhodamine-actin, or there may be a difference in the presence of barbed end capping proteins in these motile structures. This raises an additional limitation that the labeling of free barbed ends with this method does not reveal whether the labeled barbed ends are newly created, such as by ADF/cofilin severing of F-actin, or whether F-actin is not severed but barbed ends are freed by the removal of barbed end capping proteins.

Disclosures

No conflicts of interest declared.

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