Chapter 18

Measuring Retrograde Actin Flow in Neuronal Growth Cones

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Abstract

Actin flow refers to the motion of the F-actin cytoskeleton and has been observed in many different cell types, especially in motile cells including neuronal growth cones. The direction of the actin flow is generally retrograde from the periphery toward the center of the cell. Actin flow can be harnessed for forward movement of the cell through substrate-cytoskeletal coupling; thus, a key function of actin flow is in cell locomotion. In this chapter, we illustrate three different methods of quantifying retrograde F-actin flow in growth cones derived from cultured *Aplysia* bag cell neurons. These methods include tracking the movement of surface marker beads as well as kymograph analysis of time-lapse sequences acquired by differential interference contrast (DIC) imaging or fluorescent speckle microscopy (FSM). Due to their large size, *Aplysia* neuronal growth cones are uniquely suited for these methods; however, they can also be applied to any other growth cones with clear F-actin-rich peripheral domains.

Key words Retrograde F-actin flow, Actin cytoskeleton, Neuronal growth cone, Time-lapse imaging, Kymograph

Abbreviations

ASW	artificial seawater
Con A	Concanavalin A
DIC	differential interference contrast
EMCCD	Electron-multiplying gain charge-coupled device
FSM	fluorescent speckle microscopy
RBI	restrained bead interaction

1 Introduction

Neuronal growth cones are highly motile structures at the tip of growing axons and dendrites and are essential for the proper wiring of the nervous system. Growth cones sense a variety of information in the environment including molecular, mechanical, electrical, and topographical, and convert this information via signal transduction

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cascades into changes of cytoskeletal and membrane dynamics [1–3]. Thus, the neuronal growth cone is a sophisticated cellular machine that can not only detect information but also transform it into dynamic and mechanic changes that enable directional movements. These essential growth cone properties have already been recognized by Ramon y Cajal, who described this structure for the first time over 100 years ago based on his observations of numerous fixed specimen [4]. Understanding how the neuronal growth cone works is not only critical for our knowledge of nervous system wiring but also for the development of treatments to enhance axonal growth and guidance in neurological disorders such as Lissencephaly and Parkinson's disease [5] or following traumatic injury [6].

Most of our knowledge about growth cones has been derived from studies of cultured neurons from different species including mice, rats, chickens, frogs, and *Aplysia*. Neuronal cell cultures provide a number of advantages over in vivo studies, such as quantitative high-resolution imaging and control of the environment. Whereas basic physical and chemical mechanisms are likely conserved between in vitro and in vivo systems, one has to keep in mind that certain growth cone properties may be different in vivo. The *Aplysia* bag cell neuronal growth cone has served as a valuable model system for many studies due to its large dimensions, which are 5–10× the size of other growth cones. The large dimensions and highly organized cytoskeletal structures make these growth cones suitable for high-resolution live cell imaging of protein dynamics [7, 8] as well as any biophysical manipulation and analysis [9, 10].

The neuronal growth cone can be divided into three different cytoplasmic regions: the peripheral (P) domain, the transition (T) zone, and the central (C) domain. The F-actin cytoskeleton is present in all three domains, but is most evident in the P domain and T zone, especially following chemical fixation and labeling with phalloidin [11]. Microtubules are prominent in the C domain but can also penetrate into the P domain and interact with the actin cytoskeleton [12, 13], which is critical for growth cone guidance [14]. The F-actin cytoskeleton in the growth cone P domain is organized into filopodial bundles and lamellipodial networks, whereas the main F-actin structures in the T zone are represented by arcs and intrapodia [15]. All F-actin structures in the P domain and T zone undergo constant retrograde motion, whereas intrapodia also exhibit assembly-driven motility in all directions [16]. The retrograde actin flow is maintained by a continuous assembly of actin filaments in filopodia and lamellipodia pushing against the membrane at the leading edge [17], retrograde pulling by non-muscle myosin II in the T zone [18], and recycling of actin filaments in the T zone via proteins such as cofilin [19]. The rate of retrograde actin flow is determined by the rate of these three processes as well as the level of substrate-coupling. The role of

actin flow that is best understood is in adhesion-mediated growth cone advance. Adhesion molecules couple the retrograde actin flow to extracellular substrates via clutch proteins resulting in increased traction force and reduced retrograde flow [15]. Furthermore, actin flow maintains a low density of dynamic microtubules in the P domain [12]. However, there could be additional roles of retrograde actin flow that have not been characterized so far.

In this chapter, we first describe a protocol for culturing *Aplysia* bag cell neurons, which we have reported previously [20]. Then, we describe three different methods of quantifying retrograde F-actin flow in neuronal growth cones. These include tracking the movement of surface marker beads as well as kymograph analysis of time-lapse sequences acquired by differential interference contrast (DIC) imaging or fluorescent speckle microscopy (FSM). Although we have used *Aplysia* growth cones, these approaches can be applied to other growth cone preparations as well.

2 Materials

2.1 Aplysia *Bag Cell Neuronal Culture*

1. Leibovitz's L15 cell culture medium supplemented with artificial seawater (L15-ASW): L15 (powder, containing glutamine, Invitrogen #41300-039), pH 7.9, including 4 mM L-glutamine, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 9 mM $CaCl_2$, 27 mM $MgSO_4$, 28 mM $MgCl_2$, 400 mM NaCl, and 50 μg/mL gentamicin. Osmolarity should be 950–1000 mmol/kg by using an osmometer. Filter through a 0.22 μm sterile filter. Store at 4 °C for up to 1 month (*see* **Note 1**).
2. Dispase II (Sigma # 04942078001).
3. *Aplysia californica*, adult, 100–200 g, from Marinus Scientific or the National Resource for *Aplysia* at the University of Miami.
4. 0.5 M $MgCl_2$. Store at 4 °C.
5. 6 N HCl for acid-cleaning of cover glass: Mix 1 volume of H_2O ultrapure with 1 volume of 36% HCl.
6. 60 mL syringe with Luer-lock tip; 18.5 gauge sterile hypodermic needle, 2 in. long.
7. Microdissection scissors: Vannas Spring Scissors, 8.5 cm long, 7 mm blade (WPI #500086); regular dissecting scissors; two pairs of forceps: Dumont Tweezers #5—11 cm long (WPI #500341).
8. Cover glass: #1.5, 22 × 22 mm, acid-cleaned and stored in 100% ethanol.
9. 35 mm Petri dishes (Sarstedt # 83.39).

10. Poly-L-lysine (10×): 200 μg/mL Poly-L-lysine (70–150 kDa; Sigma #P-6282) stock solution is prepared in sterile H_2O ultrapure. Store at −20 °C.
11. Laminar flow bench; stereo microscope; 14 °C incubator; water bath.

2.2 Preparing Con A-Coated Surface Marker Beads

1. 1 μm silica amino beads (Bangs laboratories, # SA04000).
2. For coupling of avidin, use 1 mL of 400 μg/mL Avidin D (Vector Laboratories, # A-2000-10) in 20 mM NaP_i pH 7.0 adjusted with NaOH.
3. 20 mM NaP_i pH 7.0 for protein coupling to amino beads.
4. 8% glutaraldehyde (EM grade, Electron Microscopy Sciences, #16019).
5. 10 mM Tris–HCl pH 8.0.
6. 1 mg/mL biotinylated Concanavalin A (Con A, Vector Laboratories, # B-1005-5) in 10 mM Tris–HCl pH 8.0.
7. 0.1 M NaOH.
8. 5 mg/mL bovine serum albumin (BSA).
9. 50 mM Tris–HCl pH 8.0.
10. Table-top refrigerated centrifuge Beckman Allegra X-22, water bath sonicator, tip sonicator, End-over-end rotator, e.g., Barnstead/Thermolyne Rotisserie Labquake.

2.3 DIC Time-Lapse Imaging with and Without Surface Bead Markers

1. L15-ASW containing 5 mg/mL BSA.
2. 10 mM Tris–HCl pH 8.0.
3. 1 μm Con A beads.
4. Chamber for live cell imaging.

2.4 Fluorescent Speckle Microscopy (FSM) of Actin Dynamics

This section was adapted from [20] with permission from Springer Nature.

1. Artificial seawater (ASW): 400 mM NaCl, 10 mM KCl, 55 mM $MgCl_2$, 10 mM $CaCl_2$, 15 mM HEPES, pH 7.8. Osmolarity should be 950–1000 mmol/kg by using an osmometer. Filter through a 0.45 μm sterile filter. Store at 4 °C.
2. Imaging medium: ASW supplemented with 2 mg/mL BSA, 1 mg/mL l-carnosine, and 0.25 mM vitamin E. Filter through a 0.22 μm sterile filter. Prepare freshly on the day of the experiment.
3. Injection buffer: 100 mM 1,4-Piperazinediethanesulfonic acid (PIPES) pH 7.0 adjusted with NaOH, 1 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA). Filter through a 0.22 μm sterile filter. Store at 4 °C.

4. G-buffer: 5 mM Tris–HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM dithiothreitol, 0.2 mM ATP, 10% (w/v) sucrose. Filter through a 0.22 μm sterile filter. Prepare freshly for dilution of G-actin.
5. Alexa Fluor 488-phalloidin (for microinjection): 20 mM Alexa Fluor 488-phalloidin (Invitrogen # A12379) freshly prepared in injection buffer. Therefore, transfer a small amount (10 μL) of phalloidin dissolved in methanol into a microcentrifuge tube, remove the methanol in a speed vacuum concentrator, and reconstitute the phalloidin in injection buffer, store at −80 °C.
6. Alexa Fluor 568-G-actin (for microinjection): 1 mg/mL Alexa Fluor 568-G-actin (rabbit muscle actin; Invitrogen # A12374) freshly prepared in G-buffer; store stock aliquots of 7–8 mg/mL at −80 °C (*see* **Note 2**).
7. Imaging chamber: We used a custom-made imaging chamber consisting of a metallic holder and a plastic counterpart. Two coverslips separated by plastic spacers were positioned between the metallic and plastic holder (*see* **Note 3**).
8. Diamond pen; 22 × 2 mm plastic spacers (cut from 0.020 in. thick plastic shims); nylon grommets used as clips to hold the top and bottom part of the holder together; high vacuum grease (Dow Corning); Sparkle glass cleaner; Q-tips.
9. Microinjection needles: (Borosilicate) borosilicated glass capillaries (1B100F-4, World Precision Instruments). Microloader tips (Eppendorf #930001007).
10. Micropipette puller (Narishige PP830 or other pullers).
11. Micromanipulator: NP-2 patchman (Eppendorf); injection system: FemtoJet (Eppendorf).
12. Microscopy setup: Various combinations of imaging hardware and software are possible. The following setup has been used for most of our live cell imaging experiments: Eclipse TE2000E2 (Nikon) inverted microscope equipped with 10× objective for phase contrast imaging as well as 40× 1.3 NA oil, 60× 1.4 NA oil, and 100× 1.45 NA oil immersion objectives for DIC imaging. Flat-top motorized stage H117 ProScan™ (Prior) with linear encoders and joystick control. Electron-multiplying gain charge-coupled device (EMCCD) camera iXon Ultra 888 (Andor). X-cite 120 metal halide lamp (EXFO Photonic Solutions, Inc). Lambda 10-3 highspeed filter wheels and shutters (Sutter). Filter sets (Chroma) for rhodamine-tubulin: Tetramethylrhodamine isothiocyanate (TRITC) exciter (555/25nm) and emitter (HQ610/60 nm) of a FITC/TRITC set; for Alexa Fluor 488-phalloidin: Fluorescein isothiocyanate (FITC) exciter (485/20 nm) and emitter (517/30 nm) of a FITC/TRITC set; for Alexa Fluor

568-G-actin: TxRed exciter (575/15 nm) and emitter (610/40 nm) of a FITC/TxRed/Cy5 set. MetaMorph 7.8 software (Universal Imaging). PC with Core 2 Duo processor (3 GHz, Intel).

3 Methods

3.1 Aplysia *Bag Cell Neuronal Culture*

1. Prepare fresh Dispase-II solution by dissolving 10 mg of dispase II in a solution containing 900 mL of L15-ASW and 100 mL of sterile ultrapure water. Keep at room temperature (RT).
2. Fill a 60 mL syringe with 50 mL of 0.5 M $MgCl_2$ and attach an 18.5 gauge needle. Sterilize regular dissecting scissors, microdissection scissors, and two forceps with 70% ethanol and let air-dry.
3. Place an *Aplysia* over a Styrofoam board and inject all the $MgCl_2$ solution into the body cavity. Gently rub the animal to spread the solution throughout the body. Wait 1–3 min until the animal is completely anesthetized (*see* **Note 4**).
4. Pin the head and tail of the animal to the dissection board using two needles.
5. Use forceps to lift up the skin around the abdominal cavity and open the body wall with dissection scissors. Cut the body wall opening along the side toward the head and the tail.
6. Locate the abdominal ganglion around the mantle and gill (use Figs. 4 and 5 in [21]). Use forceps to lift up the connecting nerves rostrally to the ganglion and, cut the connecting nerves 1 cm above and below the ganglion with microdissection scissors. Store the residual body of the animal at −20 °C before incineration.
7. Transfer the ganglion into the tube with dispase II solution. In a temperature-controlled water bath, digest the tissue for 15–16 h at 22 °C (*see* **Note 5**).
8. In a laminar flow bench, prepare three 35 mm Petri dishes filled with 4 mL of L15-ASW each. Adjust the medium temperature to RT and use sterile forceps to transfer the ganglion to one of these dishes. From this step on, continue working in the laminar flow bench.
9. Preparing poly-L-lysine-coated coverslips:
   (a) Use acid-cleaned #1.5 square glass coverslips (22 × 22 mm) stored in 100% ethanol (EtOH) (*see* **Note 6**). Take a coverslip with sterile forceps and flame it with a Bunsen burner. Place the coverslip inside an empty 35 mm Petri dish (*see* **Note 6**). Depending on the desired number of

neurons per cover glass, 6–12 coverslips are usually enough to plate the cells derived from one animal.

(b) Coat each coverslip with 0.5 mL of 20 μg/mL poly-L-lysine and incubate for 20 min at RT.

(c) For each coverslip, conduct three wash cycles of water addition/vacuum aspiration using 0.5 mL of sterile ultrapure water per round. After the last wash, add 4 mL of L15-ASW into each dish.

10. Spray microdissection scissors and two forceps with 70% EtOH and let air-dry. While observing through a dissection microscope, cut the ganglion through the center along the rostral-caudal axis to separate the ganglion in two halves. Then, separate the cluster from the hemiganglion and the connecting nerves, making sure to not damage the cells with the tools and maintaining the cluster in the medium all the time.
11. Using a 20 μL pipette, transfer each cluster to one of the 35 mm Petri dishes filled with L15-ASW. From this step, continue working with one cluster at the time (*see* **Note 7**).
12. Use two forceps to remove the connective tissue sheath from the cluster. Hold the cluster with one forceps and use the other forceps to push against the connective tissue sheath. Find the opening where the cluster pops out from the connective tissue sheath and completely separate the cluster from the sheath. The cluster should appear as a "berry-like" structure.
13. Use forceps to bend a yellow P20 pipette tip at a 45° angle. Then, gently suck the cluster into the tip and push it out. Start with a low shear force and increase the force as needed. Healthy cells look bright in the center of the cell body, while dead cells appear transparent against a dark background.
14. Pick up a few healthy cells with medium and transfer them onto a poly-L-lysine-coated coverslip. Avoid transferring death cells or cellular debris (*see* **Note 8**).
15. Repeat **steps 13** and **14** until you get about 20–40 live neurons on each coverslip. Plate the cells well separated but close enough to the center of the coverslip, since it is difficult to image cells close to the edges with an oil-immersion objective. Then, remove dead cells or debris from the coverslips by picking them up with the pipette and, repeat **steps 12–15** for the second cluster.
16. After all healthy cells have been plated, keep the dishes 2 h at RT and avoid vibrations. Then, place the dishes into a 14 °C incubator until imaging is performed. Growth cones develop at neurite tips usually within 4–10 h after plating, but they get smaller with time in culture [22]. Thus, we typically use the cells within the first 48 h after plating.

3.2 Preparing Con A-Coated Surface Marker Beads

1. Resuspend 1% wt/vol 1 μm silica amino beads in H_2O ultrapure in a microcentrifuge tube. Use 10 mg bead powder in 1 mL H_2O ultrapure resuspended by vortexing and pipetting. If beads are purchased as a solution (10%), mix 100 μL beads with 900 μL H_2O ultrapure. Wash 1% beads at least once with H_2O ultrapure. Washing means: spinning the microcentrifuge tube at 21,000 RCF for 3 min, subsequently removing the supernatant, and resuspending the bead pellet in the new solution (in this case in H_2O ultrapure).
2. Tip sonication with Branson sonicator (micro tip): Output force 3, 50%, 10 pulses. This step is not necessary for 5 μm beads, since they do not cause clumping problems.
3. If you are using H_2O ultrapure, adjust pH to 6.5–7.0 by adding 0.1 M NaOH (the amount depends on pH; usually a few μL is sufficient), and check pH with stripes by quickly dipping. Be aware that you lose beads in this step (*see* **Note 9**).
4. Water bath sonication for 2 min.
5. Check beads under the scope for mono-dispersity.
6. If beads are dispersed, add 1 mL (equal volume as beads) of 8% glutaraldehyde (EM grade, Electron Microscopy Sciences, #16019; always use a freshly opened bottle). Vortex and incubate on the rotator at least for 6 h (or overnight) at RT (*see* **Note 10**).
7. Spin beads at 21,000 RCF for 3 min, and wash 3× with H_2O ultrapure (1 mL each).
8. 1× wash with 20 mM NaP_i pH 7.0.
9. Incubate beads with 1 mL of 400 μg/mL Avidin D (Vector Laboratories, # A-2000-10) in 20 mM NaP_i pH 7.0 for 4 h at RT or overnight at 4 °C.
10. Spin beads as above, remove supernatant, and incubate in 5 mg/mL BSA, 50 mM Tris–HCl pH 8.0, 0.02 % NaN_3 for 30 min at RT for blocking. Save supernatant for analysis of coupling efficiency, if you want.
11. After this blocking step, spin the beads and resuspend them again in the blocking agent to store the beads at 1% at 4 °C.
12. Take a reasonable volume of avidin beads into a new tube, e.g., 200–500 μL. Be aware that you do not usually need large volumes of beads for cell experiments and that protein-coated beads are not stable for a long time.
13. Wash avidin beads once with 1 mL of 10 mM Tris–HCl pH 8.0.
14. Pellet beads and resuspend them in biotinylated Con A solution: 1 mg/mL biotinylated lectin in 10 mM Tris–HCl pH 8.0. Use the same volume of the ligand solution as beads were in **step 1** to do binding at 1% beads. Incubate for at least 1 h at RT on a rotator or overnight at 4 °C.

15. Always store the beads in the ligand solution from now on, since this is a non-covalent interaction (ligand will come off the beads when stored in buffer only)!

3.3 DIC Time-Lapse Imaging with and Without Surface Bead Markers

1. Observe the cells under a microscope with a 10× objective 24 h after plating. Perform a half-volume medium change with L15-ASW adjusted previously to RT. If growth cones have not yet developed, it's possible to speed up the growth process by keeping the cultures at RT for 1–2 h.
2. To take high-resolution images, place the coverslip with the cells in either a custom-made or commercial imaging chamber, for example, the Series 20 platform from Warner Instruments. We use a custom-made chamber system originally designed and developed by Paul Forscher and Stephen Smith at Yale University [20] (*see* **Note 11**).
3. Mount the chamber holder onto the microscope stage of an inverted microscope. We use a Nikon Eclipse TE2000E2. Use the 10× phase objective to locate individual bag cells. Since the number of neurons is relatively low (~20–40) compared to other neuronal culture systems, it is helpful to use a motorized stage with memory function to mark the positions of individual cells.
4. Spin the stock of biotinylated ConA-coated beads in Allegra X-22 at 21,000 RCF (FX301.5 rotor), 4 °C for 5 min.
5. Remove the ligand solution (ConA) with a pipette and transfer it to a separate tube. Resuspend beads with 10 mM Tris–HCl pH 8.0 for washing (1 mL for 200 µL of 1% beads), spin again, and resuspend the beads in 10 mM Tris–HCl pH 8.0. Use the same volume as your ligand solution to get 1% beads.
6. Prepare 1 mL of washed Con A beads diluted at 1:1000 in 5 mg/mL BSA in L15-ASW. Keep this diluted bead solution at RT and use it on the day you prepared it. Add the bead dilution to the mounted imaging chamber.
7. Switch the objective to a high-resolution DIC oil-immersion objective. Set up the microscope for DIC imaging and perform Koehler illumination. Search appropriate growth cones for imaging.
8. The optimal camera settings depend on the type of CCD camera. We use either an Andor iXon 888 Ultra or a Roper Scientific CoolSnap HQ and an exposure time of 50 msec. Time-lapse sequences of growth cone dynamics are acquired in 5–10 s intervals for 10–30 min. Time-lapse sequences can be acquired with or without beads.
9. To make sure the chamber is always filled with medium, exchange with 1–3 mL of fresh medium every 30 min by

adding medium into one side of the chamber while removing medium on the other side with a vacuum line.

10. Analyze image stacks using appropriate spatial calibrations and image analysis software.

3.4 Retrograde Actin Flow Analysis Based on DIC Time-Lapse Sequences

There are two options for actin flow analysis based on DIC time-lapse sequences. The first option involves using kymographs of DIC time-lapse sequences. The second option involves tracking the centroid position of flow-coupled beads over time. In either case, ImageJ or Fiji is used as free image analysis software.

1. *Kymograph Analysis of DIC Time-Lapse Sequences*
   1. In ImageJ, import the time-lapse sequence by File>Import>Image Sequence.
   2. Apply the proper spatial calibration: Analyze>Set scale (enter the distance in pixels and actual distance in micrometers and click okay).
   3. Blur all images by selecting Process>Smooth.
   4. Apply a convolution filter to highlight the edges: Process>−Filters>Convolve and write the following kernel:

−1	−1	−1
−1	8	−1
−1	−1	−1

   5. Blur all images again as in **step 3**.
   6. From the toolbar, select the option "Straight Line."
   7. Draw a line along a filopodium in the P domain from the growth cone leading edge to the T zone. Select a line width of 1 pixel.
   8. Create a kymograph by one of the following two methods (Fig. 1):
      (a) Image>Stacks>Reslice. Select the output spacing as your spatial calibration to get a kymograph with the *x*-axis representing space and the *y*-axis representing time (both in μm). You get a kymograph that is spatially calibrated in μm or in pixels.
      (b) b) Analyze>Multi Kymograph>Multi Kymograph. Select a line width of 3 pixels. This kymograph will be in pixel by pixel.
   9. Draw a line along features that move at a constant speed. Measure the slope of this line. This could be done with the velocity measurement tool https://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Velocity_Measurement_Tool.

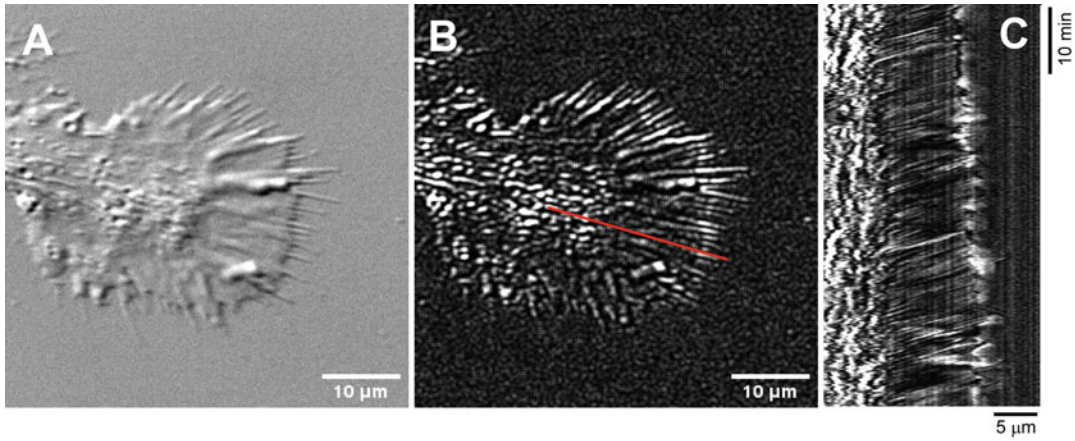

Fig. 1 Retrograde F-actin flow analysis based on DIC kymograph analysis. (**a**) DIC image of live *Aplysia* bag cell neuronal growth cone growing on coverslip coated with 50 µg/mL *Aplysia* cell adhesion molecule apCAM. (**b**) Processed DIC image as described in Subheading 3.4.1. The red line indicates where the kymograph was taken. (**c**) Kymograph of the intensity along the line indicated in (**b**). Time is going down. Scale bars as indicated

Therefore, copy the tool into the Macros/Toolsets folder and install it under Plugins>Macros>Install. It will show up as "Velocity." When you run the tool, the speed will be shown as the number of pixels along the *x*-axis (distance) divided by the number of pixels along the *y*-axis (time). This value needs to be multiplied with both the spatial (µm/pixel) and temporal calibration (pixel/min) values to get the velocity of actin flow in µm/min.

2. *Tracking Centroid Position of Flow-Coupled Beads over Time*
   1. Load the ImageJ plugin TrackMate by Plugins>Tracking>TrackMate.
   2. Import time-lapse sequence by File>Import>Image Sequence.
   3. Apply the proper spatial calibration: Analyze>Set scale (enter the distance in pixels and actual distance in micrometers and click okay).
   4. Select the frames you want to analyze in the crop settings and select the DoG detector.
   5. Set the estimated object diameter to 1 µm and the quality threshold to 100. Check the box sub-pixel localization. Click preview to verify that the desired bead has been detected by the software. If the bead of interest wasn't detected, change the quality threshold and/or object diameter. Click next and verify that the number of spots found is not smaller than the number of frames. Click "next" twice.
   6. Filter the spots based on the *x* and *y* positions. Click "next."

7. Select a simple LAP tracker, set gap closing max frame gap to 0, and let other parameters as default. Click "next" three times.
8. In the display options window, select spots and export to a . csv file. Use Excel or a script to analyze the bead position over time. An example data set taken from [23] is shown in Fig. 2.

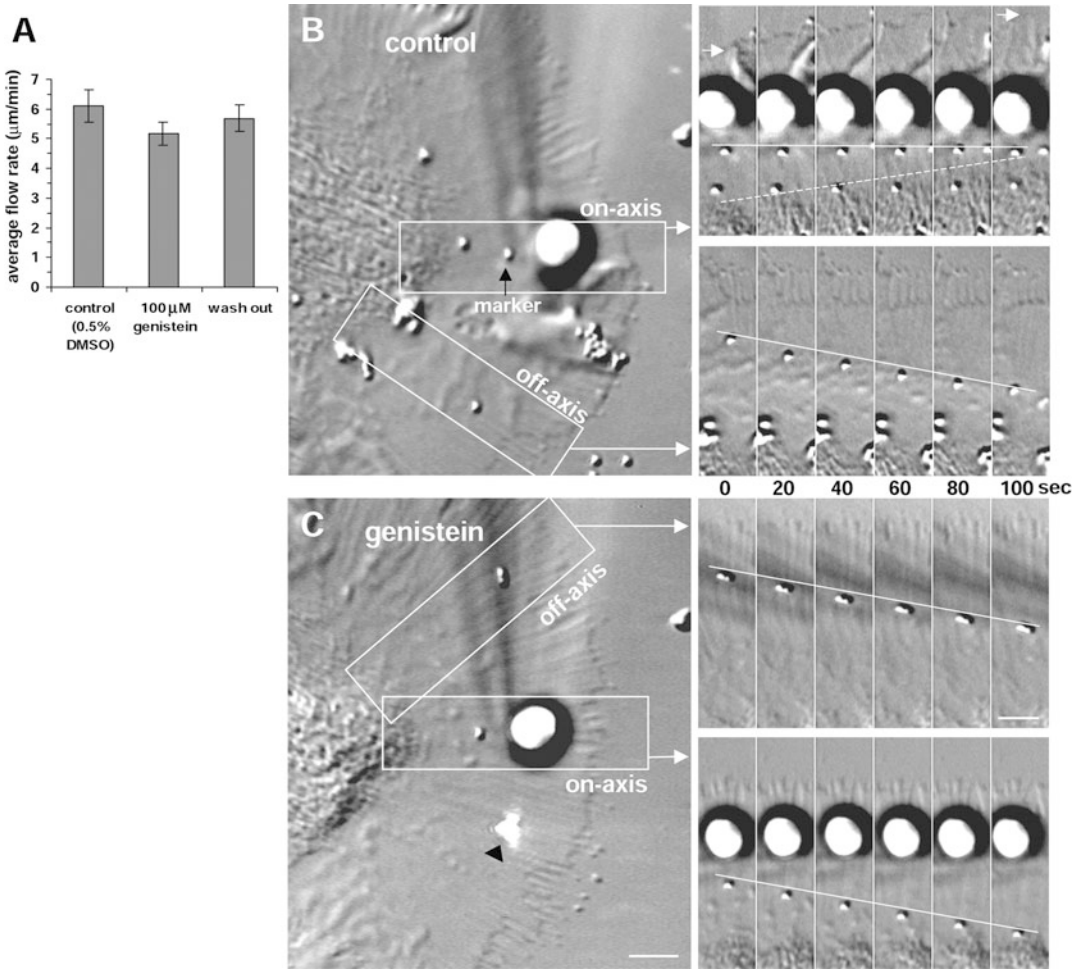

Fig. 2 Use of marker beads to determine F-actin flow in neuronal growth cones. (**a**) Quantification of retrograde F-actin flow effects by treatment with 100 μM genistein (tyrosine kinase inhibitor) for 25 min on *Aplysia* growth cones without restrained bead interactions (RBIs). Average values ± SEM. (**b** and **c**) Tyrosine kinase inhibition by genistein does not affect flow during uncoupled RBIs. All images refer to the same growth cone. Flow marker beads were placed with a laser tweezer both within the interaction corridor (on-axis) and on adjacent areas (off-axis) during an RBI under control conditions (**b**) and after pretreatment with 50 μM genistein for 20 min (**c**) Panels on the right show simultaneous DIC time sequences of on- and off-axis bead movements in areas of interests marked on the left. Flow rates for the displayed on- and off-axis beads are 0.31 versus 5.64 μm/min in control and 4.47 versus 5.14 μm/min in genistein. Central domain extension in control is indicated by dashed line and leading edge growth by a white arrow. Laser beam focus is marked with an arrowhead in **c**. Bars, 5 μm. (Reproduced from [23] with permission from Rockefeller University Press)

3.5 Fluorescent Speckle Microscopy (FSM) of Actin Dynamics

This section was adapted from [20] with permission from Springer Nature.

1. Pull needles for microinjection of cytoskeletal probes. A tip opening of 1 μm is suitable for injections into bag cell neuronal cell bodies. We use borosilicate glass capillaries and perform a double pull with all weights on the vertical puller PP830 using the heat setting "65" for the first pull and "53" for the second pull. Inspect the pulled needles on a microscope for desired tip shape before storing or using them.
2. Prepare cytoskeletal probes for microinjection as described in Subheading 2.4, **steps 5** and **6**.
3. Clarify cytoskeletal probes by centrifugation at 10,000 g for 30 min at 4 °C before microinjection. This reduces the clogging problem when performing microinjections (*see* **Note 12**).
4. Prepare your microinjection setup. We use the FemtoJet system for controlled pressure application. Start up the FemtoJet following the instructions of the manufacturer. Turn on the micromanipulator if you use a motorized one. We use an Eppendorf NP-2 patchman. Position the microneedle holder at a 40° angle to the stage.
5. Position the Petri dish containing a coverslip with cultured bag cell neurons onto the stage. Search for the position of individual neurons using a 10× phase objective and mark the cell positions.
6. Using microloader tips, backfill injection needles with a small amount of cytoskeletal probe (*see* **Note 13**).
7. Connect the microneedle to the needle holder. Keep compensation pressure at ~30–40 hPa.
8. Quickly lower the injection needle into the medium. Position the tip of the needle in the field of view without touching the surface. Switch to a 40× long working distance phase objective for microinjection and position the cell body of the neuron into the center of the field of view.
9. Check whether there is continuous flow coming out of the pipette either by using phase or fluorescence optics. A small "cloud" of fluorescent material should appear in front of the tip. If not the case, increase the compensation pressure or clear out the clog by pressing the "clean" function on the FemtoJet.
10. Position the needle tip adjacent to the cell body using the fine speed setting of the NP-2 micromanipulator. Inject the cell body by moving the needle tip quickly into an area of cytoplasm avoiding the nucleus. A fast movement increases the chance of actually penetrating the plasma membrane. An injection will result in a visible local expansion of cell volume. Leave needle in cell for ~1–3 s and then quickly move needle out since

a too large injection volume can kill the cell. Inject an estimated volume of no more than 10% of the cell body. Compensation pressure is usually sufficient for injections; if needed use an increased injection pressure.

11. To make sure the cell body was injected, switch to fluorescent mode using an appropriate fluorescent filter. A clearly injected cell will immediately exhibit fluorescent signals in the cell body that can be seen with the eyepiece.
12. After all cell bodies in one dish are injected, place the dish in a light-protected area for the cells to recover. Once all dishes are injected, carefully exchange half of the L15-ASW medium in each dish. Let the injected cells recover for at least 1 h in the dark before starting the imaging.
13. Assemble an imaging chamber as described under Subheading 3.3 and exchange the L15-ASW with imaging medium.
14. Mark cell positions with a 10× phase objective before switching to 60× or 100× oil immersion lenses to identify suitable growth cones for imaging.
15. Set up the appropriate illumination and acquisition parameters suitable for the injected fluorescent probes. We use an FITC exciter (485/20 nm) and emitter (517/30 nm) for Alexa Fluor 488-phalloidin. Reduce the fluorescence intensity with neutral density filters. The gain setting and exposure time will depend on the camera used. We typically use 500 ms exposure, gain 3, and additional EM gain on the iXon 888 ultra camera. Time intervals between images are typically 5–10 s. Healthy neurons can be imaged up to 1 h under such conditions. It is recommended to exchange fresh imaging medium every 30 min or perfuse with medium throughout the experiment. Make sure the sample remains in focus during time-lapse imaging and perform the necessary adjustments.

3.6 Retrograde Flow Analysis from Actin FSM Data

1. In ImageJ import the image sequence by File>Import>Image Sequence.
2. Apply the proper spatial calibration: Analyze>Set scale (enter the distance in pixels and actual distance in micrometers and click okay).
3. Adjust brightness/contrast by Image>Adjust>Brightness/Contrast to a level that you can see the actin signal against the background.
4. Remove noise by blurring: Process>Filters>Gaussian Blur (radius 1 on pixel basis).
5. Sharpen the images by using a sharpen filter: Process>Sharpen.
6. Select the line tool and draw a line along a filopodium in the P domain from the growth cone leading edge to the T zone. Select a line width of 1 pixel.

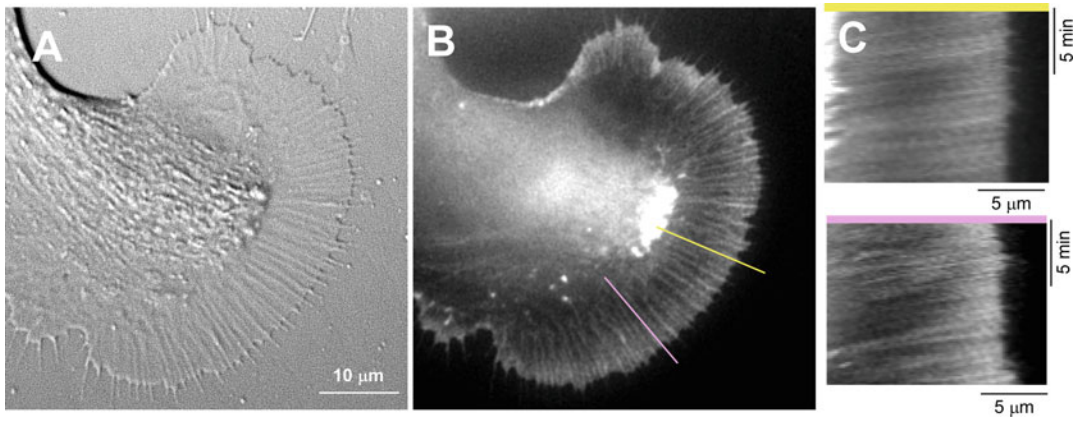

Fig. 3 Retrograde F-actin flow analysis based on actin FSM imaging. (**a**) DIC image of live *Aplysia* bag cell neuronal growth cone growing on a coverslip coated with poly-L-lysine. (**b**) Actin FSM image of the same growth cone. Cell was injected with Alexa 568 G-actin. Magenta and yellow lines indicate where kymographs were taken. (**c**) Kymographs of the intensity along the two lines indicated in (**b**). Time is going down (13 min total). Scale bars as indicated

7. Create a kymograph by one of two methods (Fig. 3):
   (a) Image>Stacks>Reslice. Select output spacing as your spatial calibration to get a kymograph with the *x*-axis being space and the *y*-axis being time (both in μm). You get a kymograph that is spatially calibrated in μm or pixels.
   (b) Analyze>Multi Kymograph>Multi Kymograph. Select a line width of 3 pixels. This kymograph will be in pixel by pixel.
8. Draw a line along features that move at constant speed. Measure the slope of this line. This could be done with the velocity measurement tool https://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Velocity_Measurement_Tool Therefore, copy the tool in the Macros/toolsets folder and install under Plugins>Macros>Install. It will show up as "Velocity." When you run the tool, the speed will be shown as number of pixels along the *x*-axis (distance) divided by the number pixels along the *y*-axis (time). This value needs to be multiplied with both the spatial (μm/pixel) and temporal calibration (pixel/min) values to get the velocity of actin flow in μm/min.

4 Notes

1. All solutions are prepared with ultrapure water.
2. Besides using commercial cytoskeletal probes, tubulin, and actin can be purified in the lab [24]. Briefly, stock solutions are prepared in the buffers described in Subheading 2.4, and

aliquots between 1 and 5 μL are stored at −80 °C after freezing in liquid nitrogen.

3. There are a number of different options for imaging chambers, either custom-made or commercial products.
4. Avoid inking and muscle contractions by handling the animal gently. Make sure not to damage the internal organs with the needle and avoid getting ink into the body cavity.
5. The effectiveness of tissue digestion depends on the temperature, time of digestion, and amount of dispase. Thus, it is advisable to optimize these variables for each batch of the enzyme.
6. Acid cleaning of coverslips can be done by incubating the coverslips in 6 N HCl for at least 1 h, followed by extensive washing with H_2O ultrapure. Then, use sterile forceps to transfer individual coverslips to a container with 100% EtOH and store them at RT.
7. Try to avoid getting bubbles between the coverslip and the Petri dish bottom. This can be achieved by adding 10 μL of H_2O ultrapure to the center of an empty Petri dish. Then, place the coverslip over the drop. In this way, you can stick the coverslip to the bottom of the Petri dish.
8. Keep the cells and the cluster in medium all the time because contact with the air interface can damage cells.
9. Glutaraldehyde activation works best under slightly acidic conditions (pH 6–7).
10. If beads are not dispersed well, do not proceed, otherwise, you will get too much clumping. Try to make the solution disperse by additional sonication. If this does not help, start with activation, and remove large, crosslinked clumps later by a low-spin centrifugation step.
11. It is essential that the two coverslips are well aligned with each other as well as with the groove of the aluminum bottom part before the top part is mounted. Uneven positioning will generate local pressure after applying the clips and result in breaking the cover glass.
12. In case of clogging, try the following: (a) spin sample at higher g forces; (b) coat needles with hexamethyldisilazane (HMDS, Pierce Chemical) by placing a few drops of HMDS on the bottom of a needle-holding box, and allow the vapor to coat the needles overnight in a fume hood; (c) clear out the clog by increasing the pressure of the microinjection system.
13. Filling about 1 cm of the narrow microloader tip provides a sufficient amount for injecting several cells. Make sure not to touch the bottom of the tube where protein aggregates could

have accumulated. Gently tap needle to remove any potential air bubbles from the solution in the microneedle. Alternative to pulling injection needles: commercial injection capillaries such as Femtotips (Eppendorf). Alternative to microloader tips: long and thin pulled glass capillaries.

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References

1. Dent EW, Gupton SL, Gertler FB (2011) The growth cone cytoskeleton in axon outgrowth and guidance. Cold Spring Harb Perspect Biol 3(3). https://doi.org/10.1101/cshperspect.a001800
2. Tojima T, Hines JH, Henley JR, Kamiguchi H (2011) Second messengers and membrane trafficking direct and organize growth cone steering. Nat Rev Neurosci 12(4):191–203. https://doi.org/10.1038/nrn2996
3. Zang Y, Chaudhari K, Bashaw GJ (2021) New insights into the molecular mechanisms of axon guidance receptor regulation and signaling. Curr Top Dev Biol 142:147–196. https://doi.org/10.1016/bs.ctdb.2020.11.008
4. Cajal R (1890) A quelle époque apparaissent les expansions des cellules nerveuses de la moelle épinière du poulet. Anatomischer Anzeiger 5:609–613
5. Munoz-Lasso DC, Roma-Mateo C, Pallardo FV, Gonzalez-Cabo P (2020) Much more than a scaffold: cytoskeletal proteins in neurological disorders. Cells 9(2). https://doi.org/10.3390/cells9020358
6. Blanquie O, Bradke F (2018) Cytoskeleton dynamics in axon regeneration. Curr Opin Neurobiol 51:60–69. https://doi.org/10.1016/j.conb.2018.02.024
7. He Y, Ren Y, Wu B, Decourt B, Lee AC, Taylor A, Suter DM (2015) Src and cortactin promote lamellipodia protrusion and filopodia formation and stability in growth cones. Mol Biol Cell 26(18):3229–3244. https://doi.org/10.1091/mbc.E15-03-0142
8. Lee AC, Suter DM (2008) Quantitative analysis of microtubule dynamics during adhesion-mediated growth cone guidance. Dev Neurobiol 68(12):1363–1377. https://doi.org/10.1002/dneu.20662
9. Athamneh AI, Cartagena-Rivera AX, Raman A, Suter DM (2015) Substrate deformation predicts neuronal growth cone advance. Biophys J 109(7):1358–1371. https://doi.org/10.1016/j.bpj.2015.08.013
10. Suter DM, Errante LD, Belotserkovsky V, Forscher P (1998) The Ig superfamily cell adhesion molecule, apCAM, mediates growth cone steering by substrate-cytoskeletal coupling. J Cell Biol 141(1):227–240. https://doi.org/10.1083/jcb.141.1.227
11. Forscher P, Smith SJ (1988) Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J Cell Biol 107(4):1505–1516. https://doi.org/10.1083/jcb.107.4.1505
12. Schaefer AW, Kabir N, Forscher P (2002) Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. J Cell Biol 158(1):139–152. https://doi.org/10.1083/jcb.200203038
13. Tanaka EM, Kirschner MW (1991) Microtubule behavior in the growth cones of living neurons during axon elongation. J Cell Biol 115(2):345–363. https://doi.org/10.1083/jcb.115.2.345
14. Tanaka E, Sabry J (1995) Making the connection: cytoskeletal rearrangements during growth cone guidance. Cell 83(2):171–176. https://doi.org/10.1016/0092-8674(95)90158-2
15. Miller KE, Suter DM (2018) An integrated cytoskeletal model of neurite outgrowth.

Front Cell Neurosci 12:447. https://doi.org/10.3389/fncel.2018.00447

16. Rochlin MW, Dailey ME, Bridgman PC (1999) Polymerizing microtubules activate site-directed F-actin assembly in nerve growth cones. Mol Biol Cell 10(7):2309–2327. https://doi.org/10.1091/mbc.10.7.2309
17. Mallavarapu A, Mitchison T (1999) Regulated actin cytoskeleton assembly at filopodium tips controls their extension and retraction. J Cell Biol 146(5):1097–1106. https://doi.org/10.1083/jcb.146.5.1097
18. Medeiros NA, Burnette DT, Forscher P (2006) Myosin II functions in actin-bundle turnover in neuronal growth cones. Nat Cell Biol 8(3):215–226. https://doi.org/10.1038/ncb1367
19. Marsick BM, Flynn KC, Santiago-Medina M, Bamburg JR, Letourneau PC (2010) Activation of ADF/cofilin mediates attractive growth cone turning toward nerve growth factor and netrin-1. Dev Neurobiol 70(8):565–588. https://doi.org/10.1002/dneu.20800
20. Suter DM (2011) Live cell imaging of neuronal growth cone motility and guidance in vitro. Methods Mol Biol 769:65–86. https://doi.org/10.1007/978-1-61779-207-6_6
21. Kandel ER (1976) Cellular basis of behavior. 727 pages.
22. Ren Y, Suter DM (2016) Increase in growth cone size correlates with decrease in neurite growth rate. Neural Plast 2016:3497901. https://doi.org/10.1155/2016/3497901
23. Suter DM, Forscher P (2001) Transmission of growth cone traction force through apCAM-cytoskeletal linkages is regulated by Src family tyrosine kinase activity. J Cell Biol 155(3):427–438. https://doi.org/10.1083/jcb.200107063
24. Waterman-Storer C (2002) Fluorescent speckle microscopy (FSM) of microtubules and actin in living cells. Curr Protoc Cell Biol Chapter 4:Unit 4 10. https://doi.org/10.1002/0471143030.cb0410s13