Chapter 13

Using Photoactivatable GFP to Track Axonal Transport Kinetics

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Abstract

The advent of photoactivatable tools has revolutionized imaging of dynamic cellular processes. One such application is to visualize axonal transport—an intricate and dynamic process by which proteins and other macromolecules are conveyed from their sites of synthesis in the cell bodies to their destinations within axons and synapses. High-quality dynamic imaging of axonal transport using photoactivatable vectors can now be routinely performed using epifluorescence microscopes and CCD cameras that are standard in most laboratories, yet this is largely underutilized. Here we describe detailed protocols for imaging cargoes moving in fast and slow axonal transport in axons of cultured hippocampal neurons.

Key words Cytosolic proteins, Soluble proteins, Slow axonal transport, Photoactivation, PAGFP, Synapsin, Neurofilaments, APP

1 Introduction

Typically, neurons have two distinct anatomical and functional domains—somato-dendritic and axonal. Axons usually extend for long distances, but the vast majority of proteins in a neuron are synthesized in the cell bodies. Therefore, neurons have intricate transport machineries that deliver proteins to distant sites within axons and synapses. Termed axonal transport, it is important to remember that this is not a developmental process, but is constitutive, occurring throughout the life of the neuron. Classic pulse-chase radiolabeling studies defined the phenomenon of axonal transport. In these experiments, radiolabeled amino acids were injected into the vicinity of neuronal cell bodies in mice in vivo [1]. These amino acids were subsequently incorporated into newly synthesized proteins in the perikarya and transported into axons by the cell's innate transport machinery. By isolating axons and examining the radiolabeled profiles at various time points after injection, researchers identified two overall "waves" of movement that they termed fast and slow axonal transport. The faster population

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moved at overall rates of 50–200 mm/day and was composed of membranous proteins; and the slower population moved only at overall rates of 2–10 mm/day and was composed of cytoskeletal and cytosolic (or soluble) proteins [2].

Even though radiolabeling studies characterized overall axonal transport, these methods could not visualize the movement, thus the identity of cargoes moving in slow and fast transport-components remained unclear. With advances in live imaging and development of fluorescent-probes, many vesicular cargoes moving in fast axonal transport have been directly visualized [3–6]. However, cargoes moving in slow axonal transport have been difficult to see. The main reason is that unlike vesicles that have discrete, punctate profiles that can be easily tracked, slow transport cargoes such as cytoskeletal and cytosolic proteins are evenly distributed in axons, precluding visualization of individual moving elements. Moreover, the latter can have freely diffusible protein pools that make optical imaging challenging. To circumvent these issues, we and others have been using photoactivatable vectors to image such cargoes. The most commonly used photoactivatable vector is PAGFP (PhotoActivatable GFP). This is a modified form of the GFP protein which has low basal fluorescence in the native state, but upon activation by violet light (405 nm), there is a 100-fold increase in its fluorescence. The basic idea is to photoactivate a small population of a given protein within an axon and to then track its dynamic behavior over time. These approaches can overcome some of the caveats mentioned above with slow-transport imaging and have proven to be useful [7–13].

Here we describe detailed protocols for axonal and somatic photoactivation of slow- and fast-component proteins tagged to PAGFP. The method uses a standard epifluorescence microscope and cultured hippocampal neurons, but can be applied to other cultured cells as well.

2 Materials

2.1 Neuronal Culture and Transfection Reagents

2.1.1 Reagents

1. Glass-bottomed dishes, uncoated (Mattek, Catalog# P35G-1.5-10-C).
2. Poly-D-Lysine (Sigma–Aldrich).
3. 0.25 % Trypsin–EDTA (Invitrogen).
4. HBSS (GIBCO).
5. D-Glucose (Sigma–Aldrich).
6. HEPES (Sigma–Aldrich).
7. Pen-Strep (GIBCO).
8. NaCl (Sigma–Aldrich).
9. Boric acid (Sigma–Aldrich).
10. Borax—Sodium Tetraborate anhydrous (Fluka).

11. B27 (Invitrogen, Catalog# 17504-044).
12. GlutaMAX (Sigma–Aldrich).
13. Fetal bovine serum (FBS, Hyclone).
14. 10× PBS (Invitrogen).
15. Opti-MEM (Invitrogen).
16. Lipofectamine 2000 (Invitrogen).
17. Laminar flow hood for sterile tissue culture conditions.
18. 5 % CO_2 incubator.
19. Hemocytometer (for cell counting).
20. 1.5 in. coverslips.
21. 70 μm filter.
22. Constructs for protein of interest tagged to PAGFP.
    (a) PAGFP–synapsin—a gift from George Augustine, Duke University.
    (b) APP:PAGFP—a gift from Christoph Kaether, Jena, Germany.
    (c) PAGFP–Neurofilament-M (PAGFP–NFM)—a gift from Anthony Brown, Ohio State University.
23. Hibernate-E low fluorescence medium (Brainbits, Catalog# HE-lf).

2.1.2 Buffers and Solutions

1. *Borax Buffer*: 51.4 mM Boric acid, 23.6 mM Borax, ddH_2O. Adjust pH to 8.5 and sterilize using a 0.22 μm syringe filter. Store at 4 °C for long-term use.
2. *HBSS* (*Dissection buffer*): HBSS, 4.44 mM D-Glucose, 6.98 mM HEPES. Adjust pH—7.3 and filter sterilize. Store at 4 °C for long-term use.
3. *Poly-D-Lysine solution*: Dissolve to obtain a final concentration of 1 mg/mL in borax buffer.
4. *Neurobasal/B27* (*NB/B27-neuronal culture media*): Neurobasal, 2 % B27, 1 % Glutamax and filter sterilized. Aliquots of NB/B27 are kept frozen at −20 °C for long-term use and at 4 °C for short-term use (up to a week).
5. *Blocking buffer*: 30 % FBS, 70 % 1× PBS, filter sterilize using a 0.22 μm syringe filter.
6. *Plating medium*: 10 % FBS, 90 % Neurobasal/B27, filter sterilize using a 0.22 μm syringe filter.
7. *Live imaging solution* (*HELF*): Hibernate-E medium, 2 mM GlutaMAX, 0.4 % D-Glucose, 37.5 mM NaCl, 2 % B27. Ensure that the components are thoroughly mixed. Filter sterilize by using a 0.22 μm filter. Aliquot and store at −20 °C. Once thawed, each aliquot can be stored at 4 °C for up to 1 week.

2.2 Live Imaging Equipment and Reagents

2.2.1 Microscope Setup

1. An inverted epifluorescence microscope (Olympus) equipped with 40× and 100× oil-immersion objectives, a dual light source fluorescence illuminator IX2-RFAW (Olympus), a CCD camera (CoolSnap HQ2 or similar), shutters IX2-SHA, a filter wheel and a pinhole.
2. Mercury lamp (HBO 100 W).
3. Violet excitation filter (D405/40, Chroma).
4. Smart Shutter (Sutter Instruments).
5. GFP cube set (Chroma, cat. no. U-N41001).
6. Dichroic mirror (T495pxr, Chroma).
7. Emission filter (HQ535/50).

The details of our microscope setup have been recently described in Roy et al. [8] and are not elaborated here. We use a custom-built device as described in Roy et al., but excellent photoactivation devices are also available commercially (Andor Technology, UK).

2.2.2 Live Imaging Setup and Solutions

1. Hibernate-E low fluorescence medium (Brainbits, Catalog# HE-lf).
2. Weather Station (Precision Control LLC) air stream incubator.
3. Vibration-isolation table (TMC).
4. Nitrogen tank.

2.2.3 Image Acquisition and Analysis Tools

1. MetaMorph imaging software (acquisition module and offline module; MolecularDevices LLC, USA).
2. MATLAB software, basic package (Math Works, USA).

3 Methods

The overall goal is to obtain cultured neurons from hippocampi of mouse (or rat) brains, allow the neurons to grow to maturity, transfect the neurons with photoactivatable vectors tagged to the protein of interest, and then study the kinetics of the photoactivated protein pools over time.

3.1 Hippocampal Neuronal Culture

1. Before dissection, add 100 µL of 1 mg/mL Poly-D-Lysine solution on to the inner well of each Mattek dish. Spread evenly and allow the coating to proceed for 2 h at room temperature. After 2 h wash the dishes thrice with ddH_2O. Dry the dishes thoroughly using an aspirator and let them sit in the hood until neurons are ready to be plated.
2. Dissect out the hippocampus from the P0–P1 mice pups after rapid decapitation using a standard dissection protocol [14]. A good tutorial video demonstrating the procedure for hippocampal neuronal culture is available on the following

website: http://www.ejnblog.org/2011/11/04/preparation-of-neuronal-cell-cultures/. Place the dissected hippocampi in ice-cold HBSS buffer before enzymatic treatment (*see* **Notes 1** and **2**). Two hippocampi from each brain yield approximately 60,000 cells after dissociation. Around 25,000 cells are plated per dish, so the number of hippocampi to be dissected needs to be calculated according to the number of dishes being plated. All dissections are carried out in ice-cold HBSS buffer.

Caution: Follow all relevant governmental and institutional ethics guidelines on the use of animals in research.

3. Once the desired number of hippocampi have been collected in ice-cold HBSS transfer them to 10 mL of pre-warmed 0.25 % Trypsin–EDTA at 37 °C. Incubate the hippocampi for 15 min at 37 °C in a water bath. After 15 min, stop enzymatic digestion by transferring the hippocampi to 10 mL blocking buffer.
4. Transfer the hippocampi from the blocking solution to 5 mL of 1× PBS. Carry out two more washes in 5 mL 1× PBS. Transfer the hippocampi to a vial containing 1 mL of plating media for dissociation (*see* **Note 3**).
5. Dissociate hippocampi in 1 mL plating media by triturating 5–8 times with an unused P1000 pipette tip. The plating media should turn slightly turbid during the dissociation process. Once this is achieved and no large chunks of tissue are seen floating in the media, the cell suspension is passed through a 70 μm filter. 10 μL of the sterile cell suspension is then used to count cells on a standard hemocytometer.

   Caution: **steps 1–4** are strictly carried out in a laminar flow fume hood to maintain sterile cultures.
6. After counting, about 25,000 cells/100 μL of the cell suspension are plated on to the center well of each poly-D-lysine coated dish. Adjust the cell dilution close to this value before plating.
7. After plating, allow the cells to recover for 1 h in an incubator (5 % CO_2, 37 °C). Then add 1.5 mL of neurobasal/B27 (NB/B27) media to the culture dish and place the dish back in the CO_2 incubator. The health of the neurons is monitored for the next 7–8 days (days in vitro; DIV7–DIV8) and 0.5 mL of fresh NB/B27 is added to each dish every 48 h up to DIV7 (*see* **Note 4**). We usually study neurons that are at least DIV7–8, with well-defined axons and dendrites.

3.2 Transfection and Live-Cell Imaging

1. A day before imaging, transfect the neurons with the desired PAGFP construct (PAGFP–synapsin, APP:PAGFP, or PAGFP–NFM) and a soluble, cytoplasmic red fluorescent protein to visualize the axons. Since the PAGFP constructs have minimal fluorescence before photoactivation, co-transfection with fluorescent markers such as monomeric red fluorescent protein (mRFP) or mCherry is essential to identify axons suitable for photoactivation.

2. To transfect five dishes, aliquot 125 μL of OPTI-MEM into two 1.5 mL Eppendorf tubes. To the first tube add 7.5 μL of Lipofectamine 2000 and mix well by pipetting. To the second tube add a total of 1.2 μg DNA of the desired PAGFP and soluble mRFP construct (in a ratio of 1:1) and mix well. Mix the contents of the two tubes and let them sit at room temperature for 15 min.
3. Meanwhile, warm NB/B27 in the water bath to 37 °C. After 15 min add 5 mL of warmed NB/B27 to the OPTI-MEM mixture. Once this is done, collect and save the conditioned medium from each culture dish so that it can be added back to the cultures after the completion of transfection. Add 1 mL of the NB/B27 OPTI-MEM mix to each culture dish to be transfected. Incubate the dishes at 37 °C, 5 % CO_2 in the incubator for 3 h with the transfection mix.
4. After 3 h, remove the transfection media from each dish and wash the cells with 1 mL fresh pre-warmed NB/B27. Remove the NB/B27 and replace with the conditioned media collected from each dish before transfection. Allow at least 4–6 h post-transfection before imaging the cells; for most experiments, we image neurons 16–24 h post-transfection (*see* **Notes 5**–**7**).
5. On the morning of imaging, turn on the stage incubator (Weatherstation) and let it equilibrate to 37 °C (this may take up to 1 h). Warm up the HELF (live imaging buffer) to 37 °C in a water bath. Now remove the NB/B27 in each transfected dish and rinse three times with 1 mL of pre-warmed HELF buffer. After the fourth rinse move the dishes to the Weatherstation and begin imaging (*see* **Notes 8** and **9**).
6. Identify transfected neurons by looking at the soluble mRFP signal, using the 40× oil immersion objective. DIV8 neurons should have several dendrites and one single axon emanating from the soma. Avoid neurons which have an overtly complex morphology, especially those in which the morphological distinction between axons and dendrites is not clear. Usually, a long straight axon with minimal morphological variations is selected as indicated in Fig. 1a. Axons are identified based on their morphology alone, and this requires some practice (*see* **Note 10**).
7. Once an axon is selected, switch to the 100× oil-immersion objective and first determine the axonal region of interest (ROI) to be photoactivated. This is done by using the soluble mRFP signal of the selected axon (*see* **Notes 11** and **12**), and an image is taken in the red channel (Fig. 1a, upper panel). The exact photoactivated ROI will depend on the instrument used. In our system (described in ref. [7]), the photoactivated area is a fixed circular ROI, determined by a pinhole situated in the optical path. In other commercial instruments (Andor Instruments),

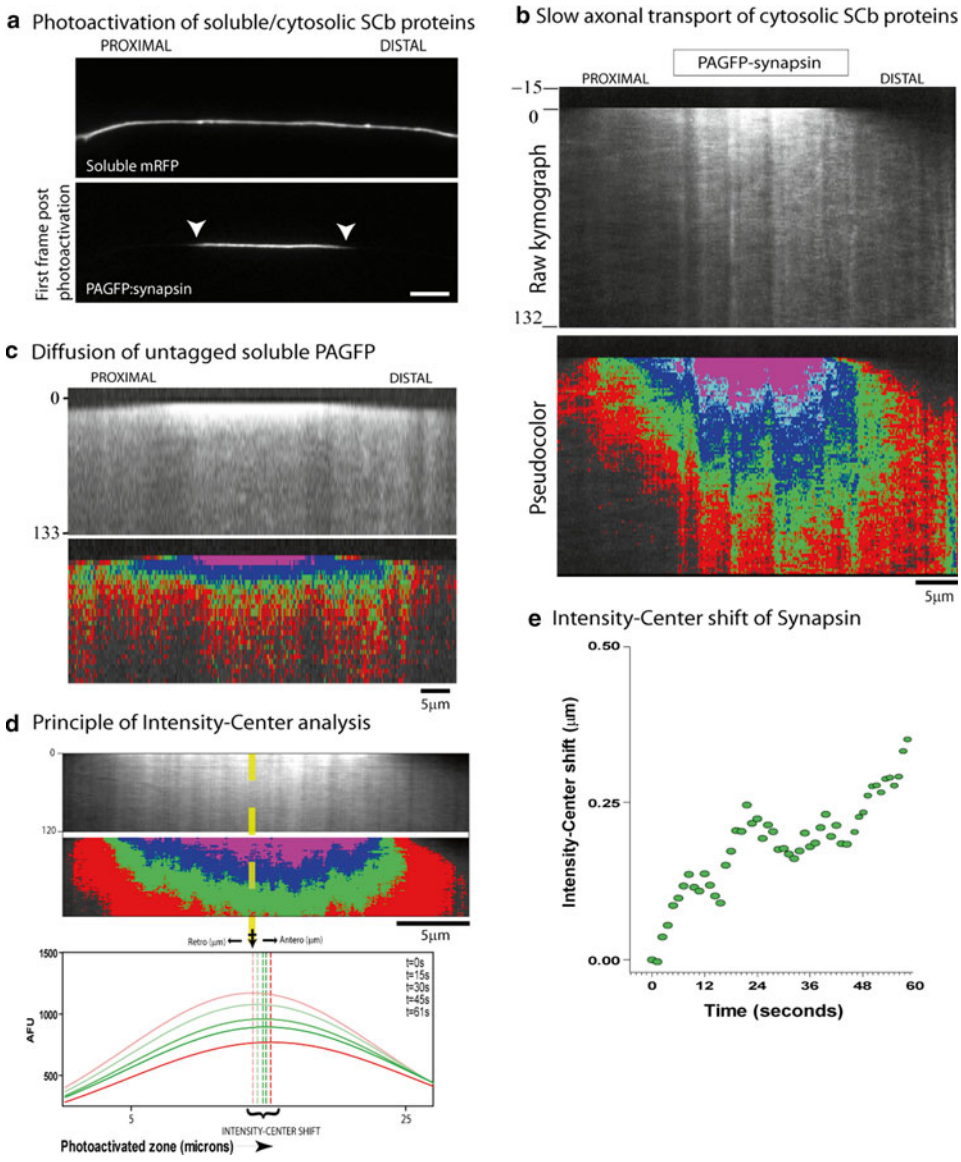

Fig. 1 Photoactivation strategy to visualize synapsin transport in axons. (**a**) Representative images of an axon co-transfected with soluble mRFP and PAGFP–synapsin showing the photoactivated GFP ROI immediately after photoactivation. *Arrowheads* denote the boundaries of the photoactivated zone. (**b**) Kymographs of photoactivated PAGFP:synapsin. Note that the synapsin fluorescence disperses bidirectionally, but has an anterograde (distal) bias. The anterogradely biased plume of fluorescence is better seen in the pseudocolor image of the kymograph above, where intensity ranges are represented by colors (*red*: lowest intensity bin, *pink*: highest intensity bin above background). Elapsed time in seconds shown on *left*, scale bar is 5 μm. (**c**) Unlike the slow, anterogradely biased flow of synapsin, the free diffusion of untagged PAGFP is very rapid and unbiased. Elapsed time in seconds is shown on *left*, scale bar is 5 μm. (**d**) Principle of the intensity center shift assay used to analyze axonal transport of soluble (SCb) proteins. PAGFP:synapsin kymographs (*above*), and its quantification (*below*). The *curves* below are overlays of selected line-scan intensities from successive frames in the PAGFP:synapsin movie that was used to generate the kymograph above. The *dashed vertical lines* mark the center of mass (centroid or "intensity-center") of the line scan fluorescence. Note the anterograde shift in the intensity-center over time. Gaussian curves are shown for clarity, but raw data are used for actual calculations. *AFU* arbitrary fluorescence units. (**e**) The "intensity-center shift" curve for the example shown in (**d**). The numerical intensity-center value in each frame of the movie is plotted over elapsed time; note the anterograde bias of the fluorescent population. Images adapted with permission from Scott et al. [9]

almost any desired region or shape can be photoactivated. Once this is done, the ROI in the axon is ready for photoactivation.

8. For PAGFP, photoactivation is carried out by violet (405 nm excitation wavelength) light. The goal here is to photoactivate a subpopulation of molecules, so that they can be visualized reliably. The time of photoactivation for the sample is important, as less than optimal activation would produce a poor signal, whereas excessive exposure of the sample to incident light may cause bleaching, or even toxicity. Accordingly, the exact time for which a sample needs to be activated has to be empirically determined by the user (*see* **Note 13**). In our setup (using a 100 W mercury lamp), we typically use 1 s of photoactivation to obtain a 10-bit GFP image for PAGFP–synapsin (Fig. 1a, lower panel). Following this, we acquire a movie which captures the movement of the activated protein over time. To track PAGFP–synapsin movement, we typically collect ~30–65 s movies of 60–130 frames, at an interval of 500 ms using 2 × 2 camera binning (*see* **Note 14**). The movies are then used to generate a line scan kymograph with a built-in function in MetaMorph. A line scan kymograph is a tool to depict motion of molecules in a linear structure (axon in this case). In our kymographs, time is depicted on the Y-axis and distance on the X-axis. A greyscale kymograph of the photoactivated PAGFP–synapsin subpopulation and its corresponding pseudocolor heat map are shown in Fig. 1b. Note the anterograde bias in the PAGFP–synapsin fluorescence over time. In contrast, soluble PAGFP disperses bi-directionally, without any bias (Fig. 1c). An intensity center shift assay was developed to calculate the rate of movement of the photoactivated protein along the axon (Fig. 1d, *see* Subheading 3.3 for details of this analysis). The overall rate of movement for synapsin (0.1–0.3 μm/s) population (Fig. 1e) is strikingly similar to rates seen with radiolabeling experiments [9].
9. In addition to axons, neuronal cell bodies can also be photoactivated using similar protocols. Figure 2 shows experiments where neurons were transfected with either PAGFP-tagged neurofilament M or APP:PAGFP and the perikarya were selectively photoactivated. Thereafter, the egress of photoactivated neurofilaments (Fig. 2a) was visualized in the dendrites. Kinetics of photoactivated APP:GFP vesicles in soma and axons can also be seen by these methods (Fig. 2b, c). The movement of individual GFP-tagged APP particles can be tracked along the axon by live imaging as indicated by the colored arrows on the line scan kymograph (Fig. 2c).

3.3 Image Analysis

1. For photoactivated proteins where individual GFP-tagged particles are seen post photoactivation (APP-GFP) a simple line scan kymograph is generated using the built-in function in

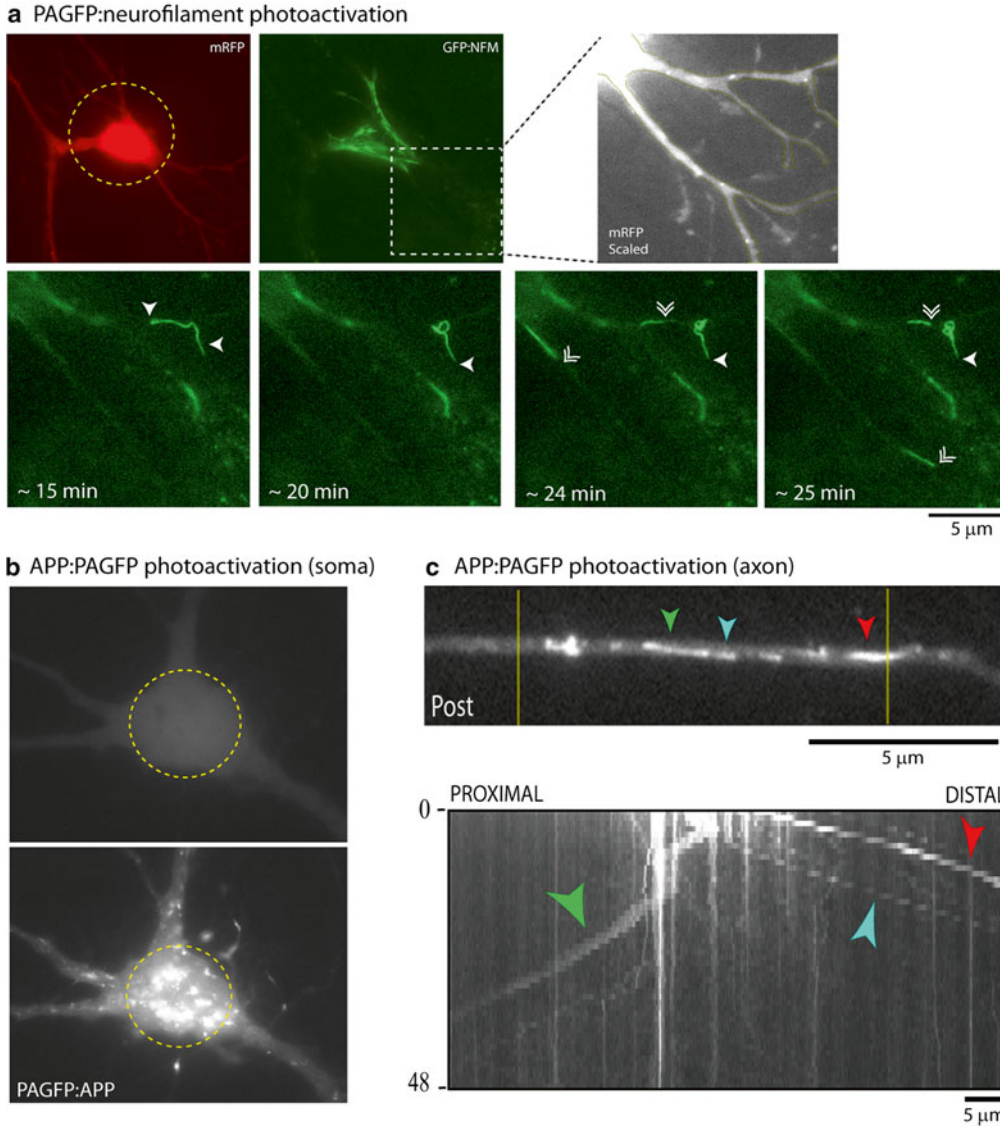

Fig. 2 Visualizing neurofilament and APP movement after somatic photoactivation. (**a**) *Top left panels*: Neurons were transfected with soluble mRFP and PAGFP–neurofilament-M (NFM), and the neuronal cell body was specifically photoactivated (*dashed circle* in mRFP image represents photoactivated ROI at 40× magnification). Note the expected filamentous appearance of GFP:NFM in the image immediately after activation. The *boxed* region was selected for dynamic imaging, and the *bottom panels* show selected frames from the corresponding time-lapse movie (the scaled mRFP image on *upper right* shows the emerging dendrites from this cell that were imaged). *Bottom panels*: Sequence of images from a time-lapse movie of the dendrites within the boxed ROI above; elapsed time after photoactivation is shown on the *bottom left. Arrowheads* mark several neurofilaments entering into the dendrites from the cell body. *Single arrowheads* mark a filament that entered into the top dendrite and paused for several minutes (note the elastic "curling" of the lagging end during the pause). The *double-arrowheads* mark other neurofilaments that moved during this time-lapse movie. Note the infrequent and intermittent nature of this transport in dendrites, as described by Brown and colleagues in the axon [11]. (**b**) Somatic photoactivation of PAGFP:APP (*dashed yellow circles* represent photoactivated ROI before and after photoactivation at 100× magnification). Note the egress of APP vesicles into dendrites. (**c**) Axonal photoactivation of PAGFP:APP, *vertical yellow lines* mark photoactivated ROI at 100× magnification (*upper panel*). Several discrete vesicles are photoactivated, some marked by *arrowheads*. In the kymograph below, note the vectorial movement of discrete vesicles (elapsed time in seconds shown on *left*). Scale bars, 5 μm

Metamorph (Fig. 2c, lower panel). Such kymographs provide sufficient information about the movement of labeled particles along the axons/dendrites—for instance the slope of the moving particles represents their velocities, where steeper slopes indicate higher velocities. For example, *see* the analyses in Tang et al. [6].

2. For soluble proteins like synapsin where fluorescent molecules disperse as a plume of fluorescence, a combination of imaging functions in Metamorph and custom written code in Matlab are used to determine the overall bias along an axon. The main goal here is to measure the position of the fluorescence intensity-peak (centroid) along the axon, for every frame of a given movie. If there is a bulk movement of fluorescence, then there would also be a corresponding shift in the centroid (*see* Fig. 1d and ref. [7] for more details). To measure the centroid, first, determine the boundaries of the photoactivated zone by thresholding the first frame of the time-lapse image series. Use a standard drop-down function in Metamorph to generate average intensity kymographs. This kymograph has information about each successive X-axis and Y-axis coordinates which carry information about the time points of imaging and successive distance from the proximal region of the axon. This generates a time distance plot which compresses the movie into a series of 2D images, facilitating further quantification.
3. Using a custom written code in Matlab basic package, the change in the center of fluorescence mass is calculated for each time point in the movie. Briefly, the center of mass for each individual horizontal line scan of a kymograph is calculated. Then the center of mass of the photoactivated region (first frame) is subtracted from the center of mass for each subsequent frame. A shift in the center of mass with each subsequent frame in either direction represents a directional bias (Fig. 1d). When line scans are drawn from the proximal to the distal direction a net anterograde bias is seen for a sample PAGFP–synapsin kymograph (Fig. 1e). For a detailed description of the analysis and videos refer to refs. [7, 9]. We can provide Matlab codes upon request.

4 Notes

1. To obtain consistent hippocampal neurons with good cell viability and transfection efficiency, we prefer using early P0-early P1 mice pups. Older pups result in higher cell death and poor viability. An alternative is to use neurons from embryonic (E18) pups.
2. During the hippocampi dissection, care should be taken to remove the fimbriae attached to the hippocampi. If not

removed properly, this results in hippocampi aggregating during dissociation, which leads to a lower cell yield.

3. We recommend dissociating not more than ten hippocampi from five brains in 1 mL of plating media for best cell yields. If more hippocampi are dissected, the dissociation should be carried out in incremental amounts of plating media.
4. Neuronal cultures are fed with 0.5 mL of fresh NB/B27 per dish every 2–3 days without removing any of the old medium for the first week (DIV7). This is essential to maintain neurons in optimal health. Letting the neurons stay in the incubator for a long time without adding fresh media leads to evaporation and change in the pH of the media.
5. The amount of DNA to be transfected needs to be optimized for each PAGFP-tagged protein and soluble marker. Too much PAGFP-tagged protein leads to high basal levels of GFP fluorescence prior to photoactivation in the axons, which may cause inconsistent results after the intensity center shift analysis.
6. Imaging should be performed between 16 and 24 h post transfection for consistent results.
7. As a negative control for imaging soluble proteins by photoactivation, one can co-transfect neurons with the empty PAGFP and soluble mRFP constructs using the transfection protocol described earlier.
8. The Weatherstation (on-stage incubator) should be equilibrated to 37 °C before performing experiments and sudden changes in the temperature of the station should be avoided during the imaging session to prevent focal drift.
9. The HELF medium maintains a pH similar to NB/B27 at atmospheric CO_2 levels, eliminating the need for a CO_2 delivery system during live imaging for several hours. Neurons in HELF survive and axons continue to grow for at least 24–48 h [11, 15].
10. Rigorous selection criteria should be implemented before axons are selected for photoactivation, especially for imaging cytosolic proteins which also invariably have a diffusible fraction. Since the axon selection is carried out based on the soluble mRFP signal it is important to select axons showing an optimal amount of RFP fluorescence. It is important to exclude neurons expressing very high or very low levels of RFP, and this needs to be empirically determined by the user. In our case, we routinely determine the entire range of RFP signals from the transfected neurons in each dish and select axons with moderate fluorescence in the RFP channel.
11. We recommend selecting a region of the axon at least 200–600 μm away from the soma of the neuron.

12. Neurons with complex morphology and axons with several bends along the primary axon should be avoided. The reason is that although movement can still be seen in those axons, analysis will be difficult—particularly in the case of soluble proteins. Ideally, linear axons with a uniform diameter should be selected. The region of the axon to be photoactivated should be fully in focus with the 100× objective throughout imaging period. In case of soluble proteins, even very small defocused areas will render the time-lapse movies unfit for analysis. Also, axons with low amounts of photoactivated GFP signal should be avoided but if necessary, the photoactivation time can be increased.
13. (Intensity of photoactivated zone) vs. (time of 405 nm light exposure) curves can be generated for each PAGFP-tagged protein to empirically determine the time required for photoactivation.
14. The parameters above are specific to our settings and should be used only as a general guideline.

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