Published in final edited form as:

Nat Protoc. ; 7(1): 62–68. doi:10.1038/nprot.2011.428.

A simple photoactivation and image analysis module for visualizing and analyzing axonal transport with high temporal resolution

Subhojit Roy^{1,2}, Ge Yang³, Yong Tang^{1,2}, and David A Scott^{1,2}

¹Department of Neurosciences, University of California, San Diego, La Jolla, California, USA

²Department of Pathology, University of California, San Diego, La Jolla, California, USA

³Department of Biomedical Engineering, Lane Center for Computational Biology, Carnegie Mellon University, Pittsburgh, Pennsylvania, USA

Abstract

We describe a strategy for analyzing axonal transport of cytosolic proteins (cps) using photoactivatable GFP—paGFP—with modifications of standard imaging components that can be retroactively fitted to a conventional epifluorescence microscope. the photoactivation and visualization are nearly simultaneous, allowing studies of proteins with rapidly mobile fractions. cultured hippocampal neurons are transfected with paGFP-tagged constructs, a discrete protein population within axons is photoactivated, and then the activated population is tracked by live imaging. We show the utility of this method in analyzing axonal transport of cps that have inherent diffusible pools and distinguish this transport modality from passive diffusion and vesicle transport. the analytical tools used to quantify the motion are also described. aside from the time needed for preparation of neuronal cultures/transfection, the experiment takes 2–3 h, during which time several axons can be imaged and analyzed. these methods should be easy to adopt by most laboratories and may also be useful for monitoring cp movement in other cell types.

INTRODUCTION

The vast majority of proteins in a neuron are synthesized in the perikarya and transported into axons and synapses via axonal transport. Transported cargoes include membranous organelles, cytoskeletal elements and CPs. Early *in vivo* pulse-chase radiolabeling studies showed that, although membranous organelles were transported rapidly in a rate class called fast axonal transport, cytoskeletal and cytosolic (or soluble) proteins—defined here as proteins without membrane-spanning or membrane-anchoring domains—moved with rates that were several orders of magnitude slower in a group called slow axonal transport (reviewed in refs.1,2). CPs are conveyed as discrete radiolabeled ‘waves’ that are slowly transported over days within long axons; this movement is incompatible with diffusion,

© 2011 Nature America, Inc. All right reserved.

Correspondence should be addressed to S.R. (s1roy@ucsd.edu).

Note: Supplementary information is available via the HTML version of this article.

AUTHOR CONTRIBUTIONS S.R. and D.A.S. conceived the imaging experiments, performed the data analyses and wrote this paper. G.Y. originally suggested the use of the intensity-center analysis algorithm to reveal bulk motion, wrote the initial MATLAB codes (that were added to by D.A.S.) and helped in writing relevant parts of this paper. Y.T. performed imaging experiments, analyzed data and provided key insights during troubleshooting.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

which exponentially decays over time^{3–6}. This rate class of slow axonal transport is also called slow component-b (or SCb). Although radiolabeling studies characterized the overall nature of transport, the movement could not be visualized by these methods. With advances in live imaging, axonal transport of discrete vesicles and individual cytoskeletal polymers was visualized, resolving many mechanistic details of this movement^{7–9}. However, in the case of cytosolic cargoes, their inherent solubility precluded visualization of their overall dynamics, and molecular mechanisms dictating the transport of cytosolic cargoes remained poorly defined.

We recently resolved the transport behavior of CPs by tagging them with photoactivatable vectors and visualizing the kinetics of the population by live imaging¹⁰. In this protocol, we describe the experimental and other technical details of this strategy. These methods use imaging components that can be easily attached to a conventional epifluorescence microscope and that involve simple image analysis tools that can be adopted by most laboratories. Although our focus is on slow axonal transport of CPs, in principle, these methods can be used to visualize/analyze the mobility of CPs in any cell type with a relatively flat morphology (Ptk-2 cells, for instance) and they may also be useful for biophysical studies of diffusion within various cellular compartments.

Comparison with other methods

These studies were originally inspired by experiments from Anthony Brown's laboratory (Ohio State University) that visualized the axonal transport of neurofilaments. The authors used a conventional setup (with no dual light source illuminator), and the photoactivation and visualization were sequential, separated by a few seconds¹¹. Although the sequential imaging setup described by Trivedi *et al.*¹¹ was appropriate for visualizing the infrequent and stochastic movement of neurofilament polymers, such temporal resolution is inadequate for studies involving proteins with highly mobile fractions (such as CPs). Although photoactivation is also achievable using commercial laser-scanning confocal microscopes, the photomultipliers used in such systems are noisy and provide limited dynamic range. As quantification of subtle fluctuations in fluorescence intensity levels is crucial to the success of these experiments, low signal-to-noise ratios are detrimental to robust transport analyses, and the high dynamic range offered by charge-coupled device (CCD) cameras is advantageous. Besides, confocal systems are not commonly available in individual laboratories, and an epifluorescence-based setup that does not compromise data quality may be desirable. Newer photoactivation systems such as Mosaic/FRAPPA (Andor Technology) also offer excellent temporal resolution, can be used in combination with epifluorescence microscopy, and allow the user to photoactivate user-defined regions of interest (ROIs). However, such systems require substantial financial investment and often necessitate major modifications to existing setups. Although photoactivation in our system is restricted by the geometric pattern of the pinhole, manipulating pinhole size/shape should accommodate most imaging experiments.

Experimental design

Overall, there are two steps to these experiments—setting up the equipment and performing the photoactivation experiments/analyzing the data (Fig. 1). Details of the microscopy setup are provided below. Briefly, an inverted epifluorescence microscope is attached to a commercially available 'dual-fluorescence illuminator' that has a dual light input. One input is used for photoactivation and the other is used for visualization. These two inputs allow simultaneous illumination (photoactivation) and visualization of the sample, provided appropriate filter combinations and shutters are used (Fig. 1). The filter cubes within the dual illuminator contain dichroic mirrors that are designed to simultaneously reflect two incident input beams (Fig. 1). Although there is a 20% reduction in light intensity in the

‘visualization input’ light path, this is not a problem, as attenuation of GFP light is necessary for live visualization. There is no loss of intensity along the ‘photoactivation input’ light path, allowing robust photoactivation of the sample. Note that the dual illuminator is necessary for simultaneous visualization/activation, as switching of filter components using conventional systems is too slow. Once the equipment is set up and tested, the actual experiment can be performed by carrying out the following steps.

First, hippocampal neurons are cultured using established protocols¹². We culture neurons at a density of 50,000 cells per cm² and do not add any mitotic inhibitors to suppress glial proliferation; thus, a glial feeder layer is not necessary^{10,13,14}. Under these conditions, neurons continue to grow and mature for at least 3–4 weeks and form functional synapses¹⁴. We also prefer using mouse hippocampal neurons for consistency, as some of our experiments use cultures from transgenic mice; however, results from mouse/rat neurons should be similar. Cultured neurons are plated on 35-mm Mattek dishes optimized for inverted microscopy. Neurons are grown on an ~1-cm cover slip attached to the bottom of the dish. This design is particularly suited to medium exchanges without disruption of the attached neurons, and we use these dishes throughout the experiment for both culturing and live imaging. Cultured neurons are incubated in Neurobasal/B27 (NB/B27)-based medium (see ref. 12 for recipe) in a 5% CO₂ incubator until they are ready for live imaging. All live imaging is done on a specially formulated live-imaging medium that is based on Hibernate-E, which allows imaging at atmospheric CO₂ levels (see below).

After growing for 7–8 d *in vitro*, neurons are co-transfected with a CP tagged to PAGFP (PAGFP:CP) and with a soluble (untagged) monomeric red fluorescent protein (mRFP) using Lipofectamine 2000. PAGFP emits very low fluorescence when activated at ~488-nm wavelength, which is used to excite conventional GFP; however, there is approximately a 100-fold increase in fluorescence intensity on ‘activation’ by light at ~405 nm (violet) wavelength¹⁵. Discretely activated pools of proteins can be followed over time (see ref. 16 for a comprehensive review). In our experiments, co-transfection of soluble mRFP was used to locate the transfected cell. After locating a transfected neuron, an axonal segment is selected for photoactivation and a discrete (20 µm) region of the axon is activated (at ×100 magnification). Thereafter, images of the activated zone are collected and analyzed as described below.

Limitations

This method was designed to evaluate the mobility of fluorescence within a linear environment (axon). Thus, these tools are most useful for evaluating axonal transport in thin cultured axons (with diameters of approximately 1–2 µm or less), or at the edge of flat non-neuronal cells (such as Ptk-2 cells), and cannot be directly applied to situations in which a large fraction of fluorophores are mobile in the z-plane. Also, as mentioned above, photoactivation of arbitrary ROIs is not possible using this system.

MATERIALS

REAGENTS

- Mouse hippocampal neurons (transfected with the desired PAGFP:CP construct) ! **CAUTION** Follow all relevant governmental and institutional ethics guidelines on the use of animals in research.
- Hibernate-E low fluorescence medium (Brainbits)
- B27 (Invitrogen, cat. no. 17504-044)
- GlutaMAX (Sigma, cat. no. 35050-061)

- D-Glucose solution (Sigma, cat. no. G8769)
- NaCl (Sigma, cat. no. S6191)

EQUIPMENT

- Microscope and accessories: an inverted epifluorescence microscope (Olympus) with $\times 40$ and $\times 100$ oil-immersion objectives, a dual light source fluorescence illuminator IX2-RFAW (Olympus), filters, two illuminating sources, a CCD camera (CoolSnap HQ² or similar), shutters IX2-SHA, a filter wheel and a pinhole (Fig. 1)
- Mercury lamp
- Violet excitation filter (D405/40, Chroma)
- Smart Shutter (Sutter Instruments)
- GFP cube set (Chroma, cat. no. U-N41001)
- Dichroic mirror (T495pxr, Chroma)
- Emission filter (HQ535/50)
- For live imaging: Weatherstation (precision control) air stream incubator, vibration-isolation table (TMC), nitrogen tank
- Glass-bottomed dishes, uncoated (Mattek, cat no. P35G-1.5-10-C)
- Laminar flow hood, tissue culture hood, incubators and so on
- MetaMorph imaging software (acquisition module and offline module; Molecular Devices)
- MATLAB software, basic package (MathWorks)

REAGENT SETUP

Live-imaging medium—Mix Hibernate-E medium with 2 mM GlutaMAX, 0.4% (vol/vol) D-glucose, 37.5 M NaCl and 2% (vol/vol) B27 (refs. 11,13). Ensure that the components are thoroughly mixed. Once prepared, this can be stored as 40-ml aliquots at -20°C . Once thawed, an aliquot can be stored at 4°C for up to 1 week. Shake the medium gently before each use.

PROCEDURE

Setting up the photoactivation imaging system • TIMING Variable

1. Attach the dual light source fluorescence illuminator IX2-RFAW to the rear port of the microscope and set it up according to the manufacturer's instructions. There are two inputs (Fig. 1a): a photoactivation input on the right and a visualization input on the left. The optical path of the setup is shown in Figure 1a. Our system is based on an Olympus inverted microscope (IX81-E); compatibility with other microscopes should be determined by the user.
2. Attach a mercury lamp to the end of the photoactivation input. This will be used to activate the sample.

▲ CRITICAL STEP We found that a mercury lamp gave optimal photoactivation, whereas photoactivation by xenon-based lamps is limited.

3. Place a shutter between the lamp and the violet filter. Any electronic shutter that can be controlled by the software can be used. The purpose of this shutter is to regulate the amount of violet light that reaches the sample for photoactivation; very high-speed shutters are not necessary. We use an Olympus shutter (IX2-SHA) that can also be triggered manually.
4. Assemble the filter slider (provided with IX2-RFAW) by inserting a violet excitation filter (D405/40) in the light path (ensure that the filter is appropriately oriented).
5. Assemble the pinhole for focusing the incident photoactivation beam on the sample. The diameter of the photoactivation ROI on the specimen plane = pinhole diameter/object magnification. In our case, we typically photoactivate a 15- to 20- μ m region using a $\times 100$ objective; accordingly, the pinhole diameter is 2 mm. A series of differentially sized pinholes can be tested for optimal activation of a given sample. Our pinholes were machined at a local instrumentation shop (Scripps). Commercial off-the-shelf pinholes (Griot) are too small for these experiments, although they can be custom-ordered from the company as well. An adjustable aperture can also be used, thereby providing more flexibility.

▲ CRITICAL STEP It is essential that the pinholes be precisely machined to achieve uniform photoactivation levels across the entire photoactivated ROI. Irregular edges along the pinhole edge will create uneven activation and create artifacts in intensity-center shifts.

6. Assemble the components in the light path of the visualization input as follows (Fig. 1a): attach a stable fluorescence light source. It is crucial to have a light source that has minimal intensity fluctuations, as fluctuating incident light can greatly confound quantitative fluorescence measurements over time. We used the X-CITE *exacte* (EXFO X-cite), which has an ultrastable DC lamp.
7. Attach a high-speed shutter to acquire images after photoactivation (we use Smart Shutter).
8. Assemble the filter wheel with the GFP excitation filter (HQ 480/40). This is detached from the GFP filter cube. In our case, we use only two positions within this wheel—either the GFP filter during a photoactivation experiment or an open position when imaging any other wavelength. Thus, very high-speed switching is unnecessary (we use an Olympus filter wheel U-FWR).
9. Insert a neutral density filter (we use Zeiss ND filters) in the filter slider (provided with IX2-RFAW). We typically reduce the incident light to 12%. As noted above, the intensity of the incident light is further diminished by $\sim 20\%$ while going through the IX2-RFAW prism.
10. Assemble the modified GFP cube as follows; this is a modification of a standard off-the-shelf GFP cube set (U-N41001, Chroma). First, detach the excitation filter and the dichroic mirror. Place the excitation filter in the filter wheel within the visualization input pathway (see Step 8). Replace the standard dichroic mirror (Q5051p, Chroma) with the T495pxr (Chroma). The emission filter (HQ535/50) stays the same. Ensure that the filters/dichroics are appropriately oriented. Note that optical components are engineered with precision, and extreme care must be taken (ensure that there are no fingerprints) while manipulating the filters/dichroics. The filters can also probably be ordered from Chroma with the above specifications.

▲ CRITICAL STEP The T495pxr dichroic cuts off wavelengths that are less than ~500 μm . This reflects the incident violet light (used for photoactivation), allowing it to strike the sample, and is crucial for the success of these experiments (Fig. 1).

Performing the photoactivation experiments • TIMING 6–8 h

11. *Preparation:* The day before imaging, co-transfect DIV (days *in vitro*) 7–8 neurons, plated on Mattek dishes with the desired PAGFP:CP and soluble mRFP constructs (we use Lipofectamine 2000). Image neurons within 17–24 h after transfection to avoid potential overexpression artifacts. At this point, the neurons are growing in the NB/B27 medium. On the morning of the imaging session, switch on the on-stage incubator (Weatherstation, precision control) to equilibrate the environment around the microscope stage at 37 °C (it is important to avoid thermal fluctuations of the focal plane during live imaging).
12. Take one dish out of the tissue culture incubator. Aspirate the NB/B27 medium from the dish and replace the NB-based medium with 1 ml of Hibernate-E–based live-imaging medium. Besides supporting the growth and maturation of cultured neurons, this formulation is designed to maintain the pH of the medium at atmospheric CO₂ levels (as opposed to 5% CO₂); it also precludes the use of cumbersome CO₂-delivering devices during imaging and maintains axonal growth and synaptic physiology for several hours^{11,13}. Repeat this process three more times, eventually placing the neurons in a fourth rinse of live-imaging medium. Although most photoactivation experiments will be completed within 1 h, neurons cultured in this medium can survive (with axonal growth) overnight if the temperature is maintained at 37 °C.

▲ CRITICAL STEP It is important to perform the medium exchanges quickly. As the neurons are switching between two different buffers, a slow exchange (and subsequent mixing of the two buffers) is undesirable.

13. Place the dish under the lens of the microscope and find transfected neurons using a $\times 40$ oil-immersion objective (using soluble mRFP). In these neurons, multiple dendrites emerge from the soma, with only a single axon, and we identify transfected axons by morphology, using established criteria to distinguish axons from dendrites (see ref. 10 for an example). Only primary axons emerging from the soma (not branches) should be selected for imaging, and neurons with inherently complex morphology should be avoided. Although this limits the number of neurons that can be analyzed in a given dish, it provides uniformity across the data sets, and consistent axonal transport of a variety of vesicle markers and CPs is seen (Y.T., S.R. and D.A.S., unpublished observations).
14. Using the $\times 100$ objective, first predetermine the ROI of the photoactivated zone by using the profile of the incident violet light on the focal plane (the ROI of the pinhole after switching only the violet light in a background region of the cover slip—‘violet ROI’). Now focus on a transfected axon (using the RFP), placing it at the center of a $\times 100$ field, and take an image in the RFP channel. Overlay the violet ROI on the RFP image (i.e., copy/paste the violet ROI onto the RFP image) to determine the anticipated photoactivation zone. Adjust the axonal orientation if necessary to ensure that the axonal segment is roughly linear and without large anatomic variations (varicosities or filopodia). Once a suitable axon is oriented, it is ready for activation (Fig. 2). We typically perform all imaging at $\times 100$, but other magnifications work as well.

15. Adjust the photoactivation exposure and the imaging time interval to suit specific imaging needs. For most experiments involving PAGFP:CP, we use a photoactivation exposure of 1 s (with 100% violet light) to obtain a reasonable signal-to-noise ratio (a postactivation GFP image of at least a 10-bit range) under our specific imaging conditions. Soluble (untagged) PAGFP can be easily activated with much lower exposures (200–500 ms), perhaps because soluble GFP molecules uniformly equilibrate throughout the axon and larger amounts are available for activation. Once activated, the axons can be imaged at various exposures/time intervals¹⁰.

■ **PAUSE POINT** Data can be analyzed at a later date if preferred.

? TROUBLESHOOTING

Analyzing the photoactivation data • TIMING 1–2 h

16. After the acquisition of images, calculate the directional bias of the fluorescent mass using an intensity-center (or centroid) assay. First, define the image background interactively. Thereafter, calculate the average background and subtract from each frame of the movie. Under attenuated-light conditions, photobleaching should be minimal for the first 30 frames as determined by photoactivation of fixed axons (see ref. 10); however, images should be corrected for bleaching if analyzed for longer time periods.
17. Threshold the first frame of the time-lapse image series to determine the boundaries of the photoactivated zone; draw a polyline across the photoactivated zone (in the proximal to distal direction of the axon) and generate average-intensity kymographs¹⁷ (using drop-down functions in MetaMorph). Each successive *x* axis and *y* axis point along the kymograph represents discrete time points of imaging and incremental proximodistal distance along the axon, respectively. Such time-distance plots, compressing the movie into 2D images, facilitate the quantification of the movement of the photoactivated fluorescent mass within the activated zone.

? TROUBLESHOOTING

18. Quantify the movement of the photoactivated fluorescent mass by calculating the change in center (or centroid) of the fluorescence mass at each successive time point of the movie, illustrated graphically in Figure 3 and in **Supplementary Video 1**. Perform all subsequent analyses of kymographs using MATLAB. Calculate the center of mass for each horizontal line scan within the kymographs as the average of pixel positions along the line scan weighted by the fluorescent intensity at each position; $R = \sum_i (r_i m_i) / \sum_i m_i$, where r_i represents *x* coordinates of equally spaced grid points along the line scan and m_i represents the intensities corresponding to these points. Thereafter, subtract the center of the mass for the photoactivated zone in the first frame from each subsequent mass center position (see Supplementary Fig. 1 for a stepwise depiction). A deviation in the center of the mass (from the original center of mass) represents a directional bias. As line scans were drawn in the proximal to distal direction along the axon, positive intensity-center shifts represent an anterograde bias for the movement of the fluorescent mass, whereas negative intensity-center shifts represent a retrograde bias (see **Supplementary Video 1** for a pictorial demonstration of the entire process). MATLAB codes are available on request.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting tips for hippocampal cultures are discussed in ref. 12. Troubleshooting guidance for photoactivation experiments is shown in Table 1.

• TIMING

Steps 1–10, Setting up the photoactivation imaging system: variable

Steps 11–15, Performing the photoactivation experiments: 6–8 h

Steps 16–18, Analyzing the photoactivation data: 1–2 h

ANTICIPATED RESULTS

The sequence of events during a typical photoactivation experiment is shown in Figure 2a. Transfected axons are identified by the presence of soluble mRFP (RFP image, far left), and a discrete population is photoactivated by these methods (GFP image, far right). Figure 2b,c shows examples from photoactivation experiments comparing cytosolic, soluble and vesicular proteins imaged using this protocol. Note that the photoactivated CP population disperses slowly as a plume of fluorescence with an anterograde bias as shown in the kymographs in Figure 2b, left. In contrast, untagged soluble PAGFP has a rapid bidirectional diffusive motion that appears unbiased (Fig. 2b, right). When a transmembrane vesicular protein is photoactivated (amyloid precursor protein (APP) is shown), individual vesicles are activated, and they stochastically depart the photoactivated zone over time (Fig. 2c), with overall kinetics that are very different from cytosolic cargoes.

Once the images are background-corrected, the intensity-center shifts are calculated from each axon using the above algorithms and plotted as means $\pm$ s.d. Typical results from the CP synapsin—known to move in slow axonal transport^{4,18}—are shown in Figure 3b (also see **Supplementary Video 1**). Note that the variations in individual data sets are comparable to those of other experiments involving fluorescent probes in neurons¹⁹. Other examples of CP transport, along with their dependence on motors/microtubules, are shown in Scott *et al.*¹⁰.

There are two key factors to be considered in obtaining quality data sets. First, it is important to photoactivate a substantial population of proteins within the axon so that its movement can be tracked with confidence. However, the extent of photoactivation from axon to axon can be variable, depending on the technical quality of transfection (and co-transfection) as well as the nature of the tagged protein. For the latter, we have found that some CPs were harder to photoactivate than others, despite using identical procedures. It was also technically difficult to consistently photoactivate a large population of vesicles using these procedures. This was likely because of variability in the distribution of vesicles along axons, but other unknown factors may also be involved. In such cases, it may be possible to photoactivate larger pools of protein by increasing the pinhole diameters and/or tagging tandem PAGFPs to the protein of interest. Second, note that the intensity-center shift algorithm assumes ideal experimental conditions (cylindrical axon, uniform photoactivation), and, although this assay is generally tolerant of minor nonuniformities in fluorescence intensities along the axon, extreme variations in intensities along the axon length and/or anatomic irregularities may abnormally weigh the curves and skew the shifts. The best results are obtained from linear, uniform stretches of axons with large photoactivated protein pools as shown in the example in Figure 2a.

Acknowledgments

S.R. acknowledges the Larry Hillblom Foundation, the March of Dimes Foundation and the University of California, San Diego Alzheimer's Disease Research Center for their support (P50AG005131), as well as G. Patterson and J. Lippincott-Schwartz for sharing the PAGFP construct.

References

1. Brown A. Axonal transport of membranous and nonmembranous cargoes: a unified perspective. *J Cell Biol.* 2003; 160:817–821. [PubMed: 12642609]
2. Roy S, et al. Axonal transport defects: a common theme in neurodegenerative diseases. *Acta Neuropathol (Berl).* 2005; 109:5–13. [PubMed: 15645263]
3. Garner JA, Lasek RJ. Cohesive axonal transport of the slow component b complex of polypeptides. *J Neurosci.* 1982; 2:1824–1835. [PubMed: 6183413]
4. Petrucci TC, Macioce P, Paggi P. Axonal transport kinetics and posttranslational modification of synapsin I in mouse retinal ganglion cells. *J Neurosci.* 1991; 11:2938–2946. [PubMed: 1715393]
5. Lasek RJ, Garner JA, Brady ST. Axonal transport of the cytoplasmic matrix. *J Cell Biol.* 1984; 99 (1 Pt 2):212s–221s. [PubMed: 6378920]
6. Koike H, Matsumoto H. Fast axonal transport of membrane protein and intra-axonal diffusion of free leucine in a neuron of *Aplysia*. *Neurosci Res.* 1985; 2:281–285. [PubMed: 2410835]
7. Kaether C, Skehel P, Dotti CG. Axonal membrane proteins are transported in distinct carriers: a two-color video microscopy study in cultured hippocampal neurons. *Mol Biol Cell.* 2000; 11:1213–1224. [PubMed: 10749925]
8. Roy S, et al. Neurofilaments are transported rapidly but intermittently in axons: implications for slow axonal transport. *J Neurosci.* 2000; 20:6849–6861. [PubMed: 10995829]
9. Wang L, et al. Rapid movement of axonal neurofilaments interrupted by prolonged pauses. *Nat Cell Biol.* 2000; 2:137–141. [PubMed: 10707083]
10. Scott DA, et al. Mechanistic logic underlying the axonal transport of cytosolic proteins. *Neuron.* 2011; 70:441–454. [PubMed: 21555071]
11. Trivedi N, Jung P, Brown A. Neurofilaments switch between distinct mobile and stationary states during their transport along axons. *J Neurosci.* 2007; 27:507–516. [PubMed: 17234583]
12. Kaech S, Banker G. Culturing hippocampal neurons. *Nat Protoc.* 2006; 1:2406–2415. [PubMed: 17406484]
13. Roy S, et al. Rapid and intermittent cotransport of slow component-b proteins. *J Neurosci.* 2007; 27:3131–3138. [PubMed: 17376974]
14. Scott DA, et al. A pathologic cascade leading to synaptic dysfunction in alpha-synuclein-induced neurodegeneration. *J Neurosci.* 2010; 30:8083–8095. [PubMed: 20554859]
15. Patterson GH, Lippincott-Schwartz J. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science.* 2002; 297:1873–1877. [PubMed: 12228718]
16. Lippincott-Schwartz J, Patterson GH. Fluorescent proteins for photoactivation experiments. *Methods Cell Biol.* 2008; 85:45–61. [PubMed: 18155458]
17. Waterman-Storer C, Desai A, Salmon ED. Fluorescent speckle microscopy of spindle microtubule assembly and motility in living cells. *Methods Cell Biol.* 1998; 61:155–173. [PubMed: 9891314]
18. Baitinger C, Willard M. Axonal transport of synapsin I-like proteins in rabbit retinal ganglion cells. *J Neurosci.* 1987; 7:3723–3735. [PubMed: 3119792]
19. Burrone J, Li Z, Murthy VN. Studying vesicle cycling in presynaptic terminals using the genetically encoded probe synaptopHluorin. *Nat Protoc.* 2006; 1:2970–2978. [PubMed: 17406557]

Figure 1. Equipment setup and experimental design. **(a)** Diagram showing the light path of visualization and photoactivation inputs (green and violet lines, respectively; see text for details). **(b)** Overall design of the experiment (see text). DIV, days *in vitro*.

Figure 2.

Photoactivation strategy to study axonal transport. (a) An example of a cultured neuron before (soluble mRFP image), during (violet photoactivation image) and after (PAGFP:synapsin photoactivated image) photoactivation. (b) Left, kymograph from the axon above shows that PAGFP:synapsin disperses as a plume of fluorescence with an anterograde bias. The dashed yellow line and hatched arrow depict the center of the photoactivated zone in these images. Right, when untagged, soluble PAGFP is photoactivated in axons, and the fluorescence disperses rapidly and bidirectionally with no bias, as expected for a diffusible probe. (c) When a vesicular marker (PAGFP:APP) is photoactivated in axons, discrete vesicles are labeled (as shown in the postphotoactivation GFP image), and individual photoactivated vesicles are transported (as shown in the kymograph below). Arrowheads in the image and kymograph represent the same vesicles. Images adapted with permission from Scott *et al.*¹⁰. Scale bars, 5 μ m.

Figure 3.

A strategy for quantifying the axonal transport of soluble proteins. **(a)** Principle of intensity bin-center analysis. Left, the intensity center of each frame is obtained by calculating the mass center of an average-intensity line scan through the axon. The kymographs above and the line scans below show that, as the fluorescent plume migrates anterogradely, there is also a concomitant shift of the intensity center (depicted by a hatched arrow in the first frame; center of photoactivation depicted by a yellow dashed line; also see **supplementary Video 1**). Right, the quantitative shift for this movie is shown as a distance/time plot. Note that any positive movement in the y axis will represent anterograde movement of the fluorescent zone. **(b)** Intensity-center shifts of PAGFP:synapsin in axons. All raw intensity-center shifts obtained (black lines) for PAGFP:synapsin, with means $\pm$ s.d. (red lines), are shown. The results on the left and right were obtained from imaging neurons at 1.2-s/0.5-s intervals, respectively. Images are adapted with permission from Scott *et al.*¹⁰.

TABLE 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
15	Poor photoactivation	Low amounts of PAGFP:CP in transfected axon	Optimize co-transfection procedures by transfecting mRFP with a GFP:CP construct. The use of soluble GFP for such optimization may not be appropriate
17,18	Huge variability and/or negative intensity-center shifts	Improper instrument setup	Check optical light path(s)
		Filopodial extensions, large protein accumulations or other anatomic variations in axons (e.g., boutons containing protein of interest or acute bends along axons)	Select linear, uniform-diameter axons with no filopodial extensions. Obtain data from at least 15–20 axons over 2–3 separate culture sets
		Sections of the photoactivated axon are out of focus	It is crucial that the entire photoactivated zone is in the focal plane throughout the duration of the experiment
		Low levels of photoactivation	Photoactivate sufficient amounts to allow robust analysis
		Selected protein has very large soluble fractions	Proteins with very large inherent soluble pools can be difficult to analyze using this technique, and applicability to a particular protein of interest must be determined empirically by the researcher