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TITLE:

Imaging and Analysis of Neurofilament Transport in Excised Mouse Tibial Nerve

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SUMMARY:

We describe fluorescence photoactivation methods to analyze the axonal transport of neurofilaments in single myelinated axons of peripheral nerves from transgenic mice that express a photoactivatable neurofilament protein.

ABSTRACT:

Neurofilament protein polymers move along axons in the slow component of axonal transport at average speeds of ~0.35-3.5 mm/day. Until recently the study of this movement in situ was only possible using radioisotopic pulse-labeling, which permits analysis of axonal transport in whole nerves with a temporal resolution of days and a spatial resolution of millimeters. To study neurofilament transport in situ with higher temporal and spatial resolution, we developed a Thy1-paGFP-NFM transgenic mouse that expresses neurofilament protein M tagged with photoactivatable GFP in neurons. Here we describe fluorescence photoactivation pulse-escape and pulse-spread methods to analyze neurofilament transport in single myelinated axons of tibial nerves from these mice ex vivo. Isolated nerve segments are maintained on the microscope stage by perfusion with oxygenated saline and imaged by spinning disk confocal fluorescence microscopy. Violet light is used to activate the fluorescence in a short axonal window. The fluorescence in the activated and flanking regions is analyzed over time, permitting the study of neurofilament transport with temporal and spatial resolution on the order of seconds and microns, respectively. Mathematical modeling can be used to

extract kinetic parameters of neurofilament transport including the velocity, directional bias and pausing behavior from the resulting data. The pulse-escape and pulse-spread methods can also be adapted to visualize neurofilament transport in other nerves. With the development of additional transgenic mice, these methods could also be used to image and analyze the axonal transport of other cytoskeletal and cytosolic proteins in axons.

INTRODUCTION:

The axonal transport of neurofilaments was first demonstrated in the 1970s by radioisotopic pulse-labeling¹. This approach has yielded a wealth of information about neurofilament transport in vivo, but it has relatively low spatial and temporal resolution, typically on the order of millimeters and days at best². Moreover, radioisotopic pulse-labeling is an indirect approach that requires the injection and sacrifice of multiple animals to generate a single time course. With the discovery of fluorescent proteins and advances in fluorescence microscopy in the 1990s, it subsequently became possible to image neurofilament transport directly in cultured neurons on a time scale of seconds or minutes and with sub-micrometer spatial resolution, affording much greater insight into the mechanism of movement³. These studies have revealed that neurofilament polymers in axons move rapidly and intermittently in both anterograde and retrograde directions along microtubule tracks, propelled by microtubule motor proteins. However, neurofilaments are diffraction-limited structures just 10 nm in diameter that are typically spaced apart from their neighbors by only tens of nanometers; therefore, the polymers can only be tracked in cultured neurons that contain sparsely distributed neurofilaments so that the moving polymers can be resolved from their neighbors⁴. Thus, it is not presently possible to track single neurofilaments in axons that contain abundant neurofilament polymers, such as myelinated axons.

To analyze the axonal transport of neurofilaments in neurofilament-rich axons using fluorescence microscopy, we use a fluorescence photoactivation pulse-escape method that we developed to study the long-term pausing behavior of neurofilaments in cultured nerve cells^{4,5}. Neurofilaments tagged with a photoactivatable fluorescent neurofilament fusion protein are activated in a short segment of axon, and then the rate of departure of those filaments from the activated region is quantified by measuring the fluorescence decay over time. The advantage of this approach is that it is a population-level analysis of neurofilament transport that can be applied on a time-scale of minutes or hours without the need to track the movement of individual neurofilament polymers. For example, we have used this method to analyze the kinetics of neurofilament transport in myelinating cultures where the axons contain abundant neurofilaments, and it is not possible to resolve individual neurofilament polymers⁶.

Recently, we described the development of a hThy1-paGFP-NFM transgenic mouse that expresses low levels of a paGFP-tagged neurofilament protein M (paGFP-NFM) in neurons under the control of the human neuron-specific Thy1 promoter⁷. This mouse permits the analysis of neurofilament transport in situ using fluorescence microscopy. In this article, we describe the experimental approaches for analyzing neurofilament transport in myelinated axons of tibial nerves from these mice using two approaches. The first of these approaches is the pulse-escape method described above. This method can generate information about the

pausing behavior of the neurofilaments, but is blind to the direction which the filaments depart the activated region, and therefore does not permit measurement of the net directionality and transport velocity⁸. The second of these approaches is a new pulse-spread method in which we analyze not just the loss of fluorescence from the activated region, but also the transient increase in fluorescence in two flanking windows through which the fluorescent filaments move as they depart the activated region in both anterograde and retrograde directions. In both approaches, parameters of neurofilament transport such as the average velocity, net directionality and pausing behavior can be obtained by using mathematical modeling of the changes in fluorescence in the measurement windows. **Figure 2** illustrates these two approaches.

This protocol demonstrates the dissection and preparation of the nerve, activation and imaging of paGFP fluorescence, and quantification of neurofilament transport from the acquired images using the FIJI distribution package of ImageJ⁹. We use the tibial nerve because it is long (several cm) and does not branch; however, in principle any nerve expressing paGFP-NFM is appropriate for use with this technique if it can be dissected and de-sheathed without damaging the axons.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University.

1. Preparation of nerve saline solution

1.1. Make 100 mL of Breuer's saline¹⁰: 98 mM NaCl, 1 mM KCl, 2 mM KH₂PO₄, 1 mM MgSO₄, 1.5 mM CaCl₂, 5.6% D-glucose, 23.8 mM NaHCO₃ in double-distilled water.

1.2. Bubble 95% oxygen/5% carbon dioxide (carbogen) through the saline solution for at least 30 minutes prior to use. Leftover saline can be reused within one week; however, it must be reoxygenated before each use.

1.3. Pour oxygenated saline into a 60 mL syringe and ensure that there is minimal air remaining in the syringe.

2. Initial assembly of nerve perfusion chamber

2.1. Connect the syringe and the tubing as shown in **Figure 1A**, placing the outflow tube into a waste flask.

2.2. Place the outer gasket into the perfusion chamber housing, ensuring that the flow inlet and outlet posts are aligned with the holes in the gasket.

2.3. Lay the inner gasket (silicone, 100 µm thick) on a #1.5 circular coverslip (40 mm diameter), carefully smoothing out any wrinkles in the gasket to ensure a tight seal. To facilitate

later assembly, place the coverslip and gasket on a paper towel or task wipe with the gasket facing up.

3. Dissection and preparation of mouse tibial nerve

3.1. Sacrifice the animal by carbon dioxide inhalation or another institutionally approved method. Start a timer when the animal ceases movement/breathing, as experiments must only be conducted within 3 hours of sacrifice⁷.

3.2. Spray the fur with 70% ethanol and remove as much as possible from the animal's legs and back using an electric razor.

3.3. Using a pair of large dissection scissors, make a dorsal incision in the skin near the middle of the spine and continue the cut around the ventral aspect of the animal. Starting from this cut, slowly reflect the skin from the legs by gently pulling it away from the muscle and cutting the fascia.

3.4. Place the animal in a supine position on a dissection tray and pin all four paws. Optionally pin the tail to reduce movement further.

3.5. Using microdissection scissors, make an incision in the thigh muscles midway between the tail and knee to expose the sciatic nerve. Ensure that the nerve, which is visible through the muscle, is not cut.

3.6. Extend the incision dorsally and ventrally to remove the muscle. Similarly, remove the muscles of the calf, keeping cuts shallow and short to avoid damaging nerves.

3.7. Remove muscles until the tibial nerve is fully exposed from the point where it branches from the sciatic nerve (at the knee) to the heel (**Figure 1B**).

NOTE: In all steps including and following dissection of the tibial nerve, avoid unnecessary exposure to ambient light to minimize possible incidental activation of paGFP in the nerve.

3.8. Grasp the tibial nerve at the spine-proximal end with a pair of forceps and cut the nerve using a pair of microdissection scissors. Taking care not to put tension on the nerve, lift it away from the muscle, cutting any attachments.

3.9. Cut the spine-distal end of the tibial nerve and transfer to a small Petri dish of room temperature oxygenated saline. From this point on in the procedure, always be certain to keep track of the proximal and distal ends of the nerve.

NOTE: One way to do this is to mark the distal end of the nerve with an angled cut such that the taper is visible.

3.10. Starting from the proximal end of the nerve, gently grasp the exposed axon ends with a pair of very fine-tipped forceps.

3.11. With a second pair of forceps, grasp the nerve sheath proximally, and slowly pull towards the distal end of the nerve. The nerve sheath will slide along the axons with minimal resistance. Ensure that no undue tension is applied to the nerve during this process.

4. Final nerve perfusion chamber assembly

4.1. Remove the nerve from the saline and quickly lay it on the circular coverslip of the perfusion chamber within the rectangular opening of the inner gasket. Grasping the proximal end of the nerve, slowly lay it down onto the coverslip under gentle tension to straighten the nerve.

4.2. Place the microaqueduct slide over the nerve with the grooved side facing the nerve, and the direction of flow parallel to the nerve so that the nerve and surrounding inner gasket are now sandwiched between the coverslip and the microaqueduct slide. Flip the coverslip and microaqueduct assembly over, and place it within the perfusion chamber housing with the microaqueduct slide apposed to the outer gasket. The nerve will now be sandwiched between the coverslip and the microaqueduct slide, which are separated by the silicone gasket, with the coverslip facing up (**Figure 1B**).

4.3. Secure the perfusion chamber by placing it in the metal housing and rotating the locking ring. Ensure that the plastic housing is fully under all metal clips and tighten well to prevent saline leakage. Overtightening may crack the microaqueduct slide or coverslip. Flip the chamber over so that the coverslip is facing down.

4.4. Slowly depress the saline syringe plunger to fill the perfusion chamber. Keep the inlet and outlet tubing, outlet flask and syringe elevated above the chamber itself at all times during setup and imaging. This avoids siphoning, which can cause introduce bubbles or cause focus instability due to negative pressure in the chamber.

4.5. Transfer the perfusion assembly to an inverted microscope stage and mount the saline syringe into the syringe pump. Start the motor at an appropriate speed for a flow rate of 0.25 mL/min. Then connect and turn on the in-line solution heater set to 37 °C.

4.6. Connect the objective heater and set to 37 °C, apply oil to the objective, and insert the perfusion chamber into the stage mount.

4.7. Apply oil to the chamber heater pad and attach to the perfusion chamber. Connect and turn on the chamber heater; set to 37 °C.

NOTE: Changes in temperature may cause bubbles to form in the perfusion chamber due to outgassing of the solution. If bubbles form, briefly increase the solution flow rate by 5-10x until bubbles clear the chamber.

4.8. Lock the perfusion chamber into the stage adapter and bring the objective oil into contact with the coverslip on the underside of the chamber.

NOTE: The Biopetechs chamber with ASI stage adapter used here are designed for an inverted microscope configuration.

5. Fluorescence activation and image acquisition

5.1. Using brightfield illumination, focus on the layer of axons on the bottom surface of the nerve closest to the coverslip surface (**Figure 1C**). Myelinated axons (typically 1 - 6 μm in diameter in adult mice) can be identified by the presence of a myelin sheath, which is visible under brightfield transmitted light illumination without contrast enhancement. Schmidt-Lanterman clefts and nodes of Ranvier are also readily apparent. Unmyelinated axons are more slender (typically $<1\ \mu\text{m}$ diameter) and are generally present in bundles (Remak bundles), where they are generally too closely apposed to be resolved from each other.

5.2. If available on the microscope, activate the auto-focus system to maintain focus over the course of timelapse imaging.

5.3. Acquire a brightfield reference image. Record the directionality of the nerve (spine-proximal and distal ends) with respect to images.

5.4. Acquire a confocal image using a 488 nm laser and an emission filter appropriate for paGFP (e.g., 525/50 nm). Keep the laser power low to minimize photobleaching, with exposure time adjusted accordingly to detect the faint signal. As an example, representative data were acquired at 5% laser power and 4 s exposures. Record the acquisition settings for use in all future experiments.

NOTE: After photoactivation, the ideal imaging settings will produce a signal-to-noise ratio > 8 and photobleaching of less than 25% of the original signal over the course of 20 images. The axons can also be imaged by widefield epifluorescence microscopy as we did originally⁷, but the image quality will be inferior due to lack of confocality.

5.5. Set the laser power to approximately 5x normal imaging power and acquire an image with an exposure time of 3-4 minutes. Though not essential, this step is recommended to bleach autofluorescence and other sources of unwanted fluorescence in order to reduce background signal and thus maximize the signal-to-noise of the photoactivated fluorescence.

5.6. Acquire an image with the settings used in step 5.4 to record the pre-activation autofluorescence after this bleaching step.

5.7. On the brightfield image, draw a line parallel to the axons with a length equal to desired activation window size. The size of this window will vary depending on the experimental goal and parameters, but typical sizes are 5 μm for the pulse-escape paradigm and 40 μm for the pulse-spread paradigm.

5.8. Using this line as a guide, draw a rectangular region of interest (ROI) across the field of view perpendicular to the axons. The region must encompass all the axons to be photoactivated.

5.9. Determine optimal settings for photoactivation with 405 nm illumination.

NOTE: Only perform this step and sub-steps prior to first experimental activation. Over the course of an experiment, the same photoactivation settings must be used.

5.9.1. Activate a region of interest repeatedly using the 405 nm laser line, low laser power (e.g., 5%), pixel dwell time (e.g., 40 μs), and one pulse, acquiring an image of the activated GFP fluorescence after each activation. Repeat until the fluorescence no longer increases, and then quantify the fluorescence in a region of interest for each image.

5.9.2. Plot the average fluorescence intensities versus pulse number. Select the number of pulses after which fluorescence no longer increases as the optimal setting for activation.

5.10. Activate the paGFP fluorescence in this region by patterned excitation with 405 nm light. Ensure that an image is acquired just prior to and just following activation.

NOTE: The ideal paGFP activation will produce a clearly defined region of fluorescence with sharp boundaries contained within the ROI.

5.11. Start a 1 minute timer as the activation finishes. At the end of 1 minute, start acquisition of a timelapse series.

NOTE: The 1 minute delay is necessary to allow for the increase in fluorescence that is observed following photoactivation of paGFP¹¹. For the pulse-spread method, a 5 minute acquisition period with 30 second timelapse intervals is sufficient for measurement of the initial slopes in the central and flanking windows to measure velocity and directionality. For the pulse-escape method, an acquisition period of 30-150 minutes with 5 or 10 minute timelapse intervals permits analysis of the long-term pausing behavior of the filaments

5.12. Save all images acquired, as well as the ROI used for fluorescence activation.

5.13. Move to a new region of the nerve and repeat steps 5.1-5.11. If the new region is along the same axon, it must be at least 500 μm from the previously activated region to avoid

detection of fluorescent neurofilaments that moved out of the other activated region. The acquisition of the final timelapse must finish before the end of the 3 hour window.

NOTE: It is possible that the preparation may be viable for longer than 3 hours, but we have not confirmed that. With proficient dissection and preparation, between five and eight 10-minute timelapse image sets may be acquired within this 3 hour window.

5.14. After the final timelapse image series is acquired, stop the flow of saline, disconnect the solution and chamber heaters, and remove the perfusion apparatus from the microscope stage.

6. Flatfield and darkfield image acquisition

6.1. Make a solution of fluorescein by adding 250 mg of fluorescein powder to 0.5 mL of double distilled water. Mix until there are no visible particles and spin the solution for 30 seconds in a tabletop centrifuge to sediment any undissolved material. This solution can be stored for several months at 4 °C if protected from light exposure.

6.2. Add 8 µL of fluorescein solution to a slide and apply a #1.5 coverslip. Blot excess liquid, seal with nail polish, and allow to dry.

NOTE: At this high concentration, the strong absorption of the fluorescein dye extinguishes the illuminating beam within the solution, producing a thin plane of fluorescence at the surface of the coverslip that is both uniform and resistant to photobleaching due to rapid diffusive exchange¹².

6.3. Place the fluorescein slide coverslip-side down on the inverted microscope stage and adjust the focus on the thin plane of fluorescence at the surface of the coverslip. Move around the slide to find a field of view that does not contain air bubbles (dark spots) or large fluorescein particles (bright spots).

6.4. Acquire a z-stack spanning 6 µm at 0.2 µm intervals such that the middle image is the original focus plane. Use a short exposure time (e.g., 40 ms) because the fluorescence will be very bright. This z-stack acquisition is necessary to capture the maximal fluorescence across the field of view as the coverslip is rarely perfectly horizontal and the plane of fluorescein fluorescence is very narrow. Repeat this for a total of 25 fields of view, moving the stage by at least 20 µm in any direction between images.

6.5. Close all light path shutters, including the camera shutter, and set the laser power and the exposure time to 0 s. Acquire a stack of 100 images with these settings. These images will be averaged to generate the darkfield image, which will be used to correct for dark current and the bias offset on the camera chip.

NOTE: Streaming acquisition is an ideal way to capture these images.

7. Imaging glycolytically inhibited nerves for bleach correction

7.1. Make and oxygenate a saline solution as in step 1; however substitute 2-deoxy-D-glucose for D-glucose and add 0.5 mM sodium iodoacetate to inhibit glycolysis¹³. We refer to this as “inhibitory saline”.

7.2. Repeat steps 2-5 using the inhibitory saline, with a 10-30 minute timelapse image set in step 5.11. Allow 40-50 minutes after application of inhibitory saline before imaging to ensure complete inhibition of neurofilament transport.

NOTE: Glycolytic inhibition will eventually kill the axons so there is a narrow window of time after inhibition in which to acquire data, typically about 30 minutes. An indicator of the level of metabolic inhibition is the flavin autofluorescence of the axonal mitochondria, which can be detected in the timelapse series due to the long exposures that we use to image the paGFP fluorescence¹⁴. Typically, mitochondrial autofluorescence will become readily apparent by 70-80 minutes after applying inhibitory saline and increases over time. If the mitochondria begin to round up or fragment, and then cease imaging.

8. Image processing and analysis using ImageJ

8.1. Flatfield and darkfield correction

8.1.1. Open the darkfield image stack and average the images by clicking **Image | Stacks | Z Project** and selecting **Average Intensity** in the drop-down menu to generate the **darkfield** image.

8.1.2. Open the fluorescein flatfield image stacks and create a maximum intensity projection of each (25 in total) by clicking **Image | Stacks | Z Project** and selecting **Max Intensity** in the drop-down menu.

8.1.3. Combine the resultant 25 maximum projection images into one stack by clicking **Image | Stacks | Images to Stack**. Create an average intensity projection of this stack by clicking **Image | Stacks | Z Project** and selecting **Average Intensity** from the dropdown menu to generate the **flatfield image**.

8.1.4. Subtract the darkfield image from the flatfield image by clicking **Process | Image Calculator**, selecting **Subtract** as the operation. Ensure that the **32-bit (float) result** option is checked. The result is the **corrected flatfield image**.

8.1.5. Measure the average pixel intensity of the corrected flatfield image by first clicking **Analyze | Set Measurements** and checking the **Mean gray value** box, and then pressing the ‘m’ key.

8.1.6. Divide the corrected flatfield image by its average intensity by clicking **Process | Math | Divide** and entering the average gray value obtained in step 7.1.5. This will produce the **inverse gain** image.

8.1.7. Open the pre-activation and post-activation images along with the timelapse image stack. Combine the images into a single stack by clicking **Image | Stacks | Tools | Concatenate**, and select the images in chronological order from the dropdown menus. Make sure that the **Open as 4D image** option is not selected. The resulting stack is the **full image set**.

8.1.8. Repeat step 8.1.4 on the **full image set**, and then divide the result by the **inverse gain** image by clicking **Process | Image Calculator** and selecting **Divide** as the operation. This will produce the **corrected full image set**, in which each image has been corrected for the non-uniformity in the field of illumination and on the detector.

8.2. Image stack alignment

8.2.1. To correct for misalignment of the image planes in the timelapse series due to stage or sample drift, install the **Alignment by fixed region** plugin (**Supplemental File 1**) by clicking **Plugins | Install Plugin**, navigating to the folder containing the plugin file, and selecting the plugin. Restart ImageJ after installing the plugin.

NOTE: This plugin aligns images based on the “least squares congealing” principle¹⁵.

8.2.2. Draw an ROI on the **corrected full image set** which spans several axons and does not extend beyond the proximal and distal boundaries of the activated fluorescence within each of the axons. The geometry of the region is unimportant, however excluding areas in which structures change shape or size will improve alignment.

8.2.3. Run the alignment plugin by clicking **Plugins | Alignment by fix region**. The plugin places a default 2-pixel maximum on displacement between frames, however this may be adjusted in the initial popup window if there is significant drift of the sample. The alignment may take several minutes, depending on the size of the image stack.

8.2.4. Visually inspect the aligned stack to assess the quality of the alignment. Some frames may then need to be aligned manually, as the automated alignment may not function well for large fluctuations in fluorescence between frames. This may be accomplished while viewing a frame which must be shifted by clicking **Image | Transform | Translate**. Click **No** on the following popup asking whether the entire stack should be translated.

8.2.5. Save this as the **aligned full image set**. Use only integer pixel values in translation and ensure that the dropdown interpolation menu is set to **None**, as fractional pixel shifts or interpolation will alter the data due to resampling of the pixel intensities.

8.3. Measurement of fluorescence intensities

8.3.1. Draw an ROI using the Angle tool on the first frame of the **aligned full image set** with the first arm along one edge of the activated region, perpendicular to the axons, and the second arm vertical. Press the 'm' key to measure the angle, which describes the orientation of the axons in the field of view.

8.3.2. Set the scale of the images so that dimensions are measured in microns by clicking **Analyze | Set Scale** and entering the appropriate values.

8.3.3. Open the ROI manager by clicking **Analyze | Tools | ROI Manager**. For pulse-escape paradigm, skip to step 8.3.7. For pulse-spread, continue to step 8.3.4.

8.3.4. Draw a square ROI of any dimensions, and then click **Edit | Selection | Specify**. Ensure that the **Scaled** units option is checked, and then set the ROI to a width of 15 μ m and a height equal to or greater than the height of the image.

8.3.5. Rotate the ROI by the angle measured in step 8.3.1 by clicking **Edit | Selection | Rotate** to make it perpendicular to the axons, and place the ROI with one side along the proximal edge of the activated region. Add this ROI, which we will refer to as the proximal guide ROI, to the manager by pressing the 't' key.

8.3.6. Drag the ROI to align with the distal edge of the activated region and add to the ROI manager again by pressing the 't' key. We will refer to this as the distal guide ROI. The proximal and distal guide ROIs will be used later to draw the flanking measurement ROIs.

8.3.7. Select axons for quantification, using the timelapse image, acquired in step 5.10 above. This image is helpful for this purpose because it captures the weak autofluorescence of the axons, revealing their morphology outside of the activated region.

NOTE: Axons that do not meet the following criteria are excluded from the analysis:

- Axons must be in focus along the entire length of all measurement windows.
- Axons must be within 5° of perpendicular to the edges of the activation region.
- Axons must have no invaginations within 5 μ m of the edges of the activation region.
- Exclude axons which change shape visibly during the course of imaging.
- Exclude axons that appear unhealthy as evidenced by the absence of a discrete activated region in the post-activation image (**Figure 1D**, top), as this is indicative of diffusive dispersion of the activated fluorescence, which happens when the axon dies.

8.3.8. Observe long-exposure bleaching image for autofluorescent structures within axons. This fluorescence is due to flavins within mitochondria¹⁶. Exclude axons from analysis if these mitochondria appear rounded or fragmented (**Figure 1E**, top), as opposed to extended, linear structures (**Figure 1E**, bottom), as this is an indication of metabolic decline.

8.3.9. Using the proximal and distal guide ROIs created above, draw three measurement ROIs per axon being analyzed: a central window encompassing the axon within the 40 μm activated region, and two flanking windows with width constrained by flanking window 15 μm ROIs and height constrained by the diameter of the axon at the border of the activation region. Add all three regions to the ROI manager. For the pulse-escape paradigm, only the 5 μm central region is needed. For glycolytically inhibited axons, draw a single region no more than 5 μm wide in the middle of the activated region that does not extend outside the axon.

8.3.10. Repeat step 8.3.9 for all axons which meet criteria of steps 8.3.7 and 8.3.8.

8.3.11. Set active measurements to average pixel intensity by clicking **Analyze | Set Measurements** and selecting the **Mean gray value** option. Ensure that no other measurement options are checked.

8.3.12. Select all ROIs in the ROI Manager window by selecting the window and pressing 'Ctrl' + 'a'. In the ROI Manager window, click **More | Multi Measure** to measure the fluorescence intensities. Copy the data from the results window to a spreadsheet for further analysis.

8.3.13. Set active measurements to region area by clicking **Analyze | Set Measurements** and selecting the **Area** option. Ensure that no other measurement options are checked.

8.3.14. Repeat step 8.3.12. It is only necessary to copy one row of the results for the area, as the area does not vary with time.

9. Photobleach correction

9.1. In the data spreadsheet for glycolytically inhibited axons, subtract the mean fluorescence of frame 1 (the pre-activation frame) from the mean fluorescence of each frame starting at frame 3 (the first timelapse frame) for a given ROI. The results are the **background-subtracted means**.

9.2. Plot the data as a scatterplot with the frame numbers as the abscissa. Fit an exponential trendline to the data from each ROI (most spreadsheet programs have this function) with an equation in the form of Ae^{-bx} . This equation is equivalent to the photobleaching function $F_t = F_0 * e^{-t\gamma}$ where F_0 is the fluorescence at the first frame of the timelapse, γ is the exponential bleaching rate, t is the time, and e is the natural logarithm base.

9.3. Repeat steps 9.1.1-9.1.2 for all ROIs of all axons from the glycolytically inhibited nerves. For the most accurate estimate of the photobleaching rate, use at least 15 axons in total from at least 5 separate nerves. Use the average of the exponential bleaching rates (γ) from all inhibited axons to correct the experimental data for photobleaching. A new bleaching calibration must be performed for each experiment or study, because photobleaching is dependent on the image acquisition settings and the laser power, which can change over time.

9.4. Repeat step 9.1.1 for all regions of axons imaged with normal saline (i.e. not glycolytically inhibited).

9.5. Divide each data point by $e^{-\gamma t}$, using time for t and the average γ found in step 9.1.3. These are the **photobleach-corrected means**.

9.6. Multiply each data point by the area for that region of interest to find the **total fluorescence** in that region at each time.

REPRESENTATIVE RESULTS:

Figure 3 shows representative images from pulse-escape and pulse-spread experiments. We have published several studies that describe data obtained using the pulse-escape method and our methods for the analysis of those data^{5-8,17}. Below, we show how the pulse-spread data can yield information on the directionality and velocity of neurofilament transport, which we have not reported previously.

Neurofilament transport in the axon is intermittent and bidirectional. This transport can be described by the fraction of filaments moving at any given time in the anterograde and retrograde directions, p_a and p_r respectively, and their velocities in the anterograde and retrograde directions, denoted by v_a and v_r . If we take the total quantity of neurofilament polymer per unit length of axon to be n , and then the fluxes in the anterograde and retrograde directions through a given region are given by

$$j_a = np_a v_a$$

and

$$j_r = -np_r v_r ,$$

respectively, and the total flux j is given by

$$j = j_a + j_r = n(p_a v_a - p_r v_r) ,$$

where n has units μm^{-1} , v has units $\mu\text{m} \cdot \text{s}^{-1}$ and j has units s^{-1} . Since the flux at a given location along the axon is the quantity of neurofilament polymer that moves past that location in a unit of time, it is related to the average velocity \bar{v} through $j = n\bar{v}$. Thus, we can write

$$\bar{v} = p_a v_a - p_r v_r .$$

In a pulse-spread experiment the flux can be determined from the rate of departure of the fluorescent neurofilaments from the activated region, which we refer to as the central window. The total loss of fluorescent neurofilament polymer from this central window per second ($\frac{dQ_c}{dt}$) is the sum of the losses due to fluorescent neurofilament polymers leaving in anterograde and retrograde directions, i.e.

$$\frac{dQ_c}{dt} = -(v_a p_a + v_r p_r) n.$$

Normalized to the initial content of fluorescent neurofilament polymer in the central window, i.e. $Q_c(0) = na$, where a is the length of the central window, this rate of loss then becomes

$$\frac{d\bar{Q}_c}{dt} = \frac{-v_a p_a + v_r p_r}{a} \equiv -S_c, \quad ,$$

where $-S_c$ is the slope of the decrease in fluorescence in the central window, which is initially linear for a time period given by a/v^8 . For a 40 μm central window, this equates to just tens of seconds. However, for windows this large, the transition to the exponential decay phase is gradual and the slope is effectively linear for several minutes or more⁸.

At early times, the flanking windows capture all the neurofilaments that exit the central window because these filaments do not have sufficient time to pass through the flanking windows and exit them on the other side. In this case, the quantities of fluorescent neurofilament polymer that leave the central window anterogradely and retrogradely per second, i.e. the fluxes j_a and j_r , are given by the increase in the neurofilament content Q_p and Q_d in the flanking windows. Normalized to the initial content of fluorescent neurofilament polymer in the central window, the rates of increase in the flanking windows become

$$\frac{d\bar{Q}_p}{dt} = -j_r/na = \frac{p_r v_r}{a} \equiv S_p$$

$$\frac{d\bar{Q}_d}{dt} = j_a/na = \frac{p_a v_a}{a} \equiv S_d, \quad ,$$

where S_p and S_d are the slopes of the increase in fluorescence in the proximal and distal flanking windows, respectively.

Thus, we can express the average velocity in terms of the slopes in the flanking windows, i.e.

$$\bar{v} = a(S_d - S_p), \quad (\text{Eq. 1})$$

and the ratio of the number of anterograde and retrograde moving neurofilaments in terms of the ratio of these slopes, i.e.

$$\frac{p_a}{p_r} = \frac{S_d}{S_p} \frac{v_r}{v_a} = \frac{\gamma_{ra}}{\gamma_{ar}}, \quad (\text{Eq. 2})$$

where γ_{ar} and γ_{ra} denote the rates at which neurofilaments reverse from anterogradely to retrogradely moving and vice versa⁸. The values of v_a and v_r can be determined by measuring the movement of individual neurofilaments in cultured neurons, as reported previously¹⁷. Importantly, the expression for the velocity in Eq. 1 only applies at short times after activation during which fluorescent neurofilaments enter the flanking window but do not leave. The duration of this short time window will depend on the length of the flanking windows and the

kinetics of the neurofilament movement. The longer the flanking windows, in principle, the longer the time window. Theoretically, one can test that this criterion is met by confirming that

$$-S_c = S_p + S_d. \quad (\text{Eq. 3})$$

In contrast to the expression for the velocity, which is given by the difference between the slopes in the flanking windows, the expression for the directionality in Eq. 2 is robust to flanking window size because it is given by the ratio of the slopes in the flanking windows.

Figure 3 shows representative results from pulse-escape and pulse-spread experiments on myelinated axons in the tibial nerve of an 8-week old male mouse from our Thy1 paGFP-NFM line on a C57Bl/6J background, using window sizes of 5 and 40 μm , respectively. Mice of at least 2 weeks of age, both male and female, have been used for these experimental paradigms, and the appropriate age and sex of mice should be determined by the researcher depending on what is being tested in the study. Over time, it can be seen that the edges of the activated regions blur due to the departure of neurofilaments in both anterograde and retrograde directions, resulting in loss of fluorescence from the central window.

For the pulse-escape method (**Figure 3A, C, E**), the fluorescence decay kinetics in the activated region can yield information on the long-term and short-term pausing behavior^{8,18}. For these experiments, the activated region can be short (we typically use 5 μm ; see yellow box in **Figure 3A**). For the pulse-spread method (**Figure 3B, D, F**), we use a longer activated region (40 μm here; see yellow box in **Figure 3B**) in order to provide a larger pool of fluorescent neurofilaments. This lengthens the time over which the fluorescence increase in the flanking regions remains linear (see above). The linear domain of this fluorescence increase in the flanking windows can also be increased by increasing the length of these windows, however this is limited by the size of the field of view. We used 15 μm flanking windows, shown in red and green, for the data shown in **Figure 3D**.

Figure 3E,3F shows the quantification of the total fluorescence intensities (i.e., the sum of the pixel intensities) for the measurement windows shown in **Figure 3C,3D** respectively. For the pulse-escape method the decay is biphasic, with an initial exponential decay which represents the departure of on-track neurofilaments. This transitions at around 10-20 minutes to a second slower exponential decay that represents the mobilization and departure of off-track filaments⁸. For comparison with the pulse-spread method, we show only a 12 minute time course in **Figure 3E**, but usually the time course of the analysis needs to be longer (typically 30-120 minutes) to capture the long-term pausing kinetics^{5,6,18}. For the pulse-spread strategy, the calculations of the velocity and directionality of the movement are dependent only on the slopes in the flanking windows. For the window lengths used here, the linear phase extends for about 5 minutes. The duration of this time window of linearity should be assessed by incrementally shortening the time used to measure the slope, and determining the point at which the slope no longer increases. This duration can be increased by increasing the window lengths if the camera field of view permits, though the window lengths should be held constant for all axons in a given experiment. As an example, we have used the first 5 minutes of data to

measure the slopes for the pulse-spread data in **Figure 3F**, along with 11 other axons in the same nerve for a total of 14 axons. This results in slopes of 72 A.U./min, 594 A.U./min, and 111 A.U./min for the proximal, central and distal windows, respectively (A.U. = arbitrary units). In order to normalize these values, they are divided by the initial fluorescence of the central window, resulting in rates of 0.108 %F₀/min, -0.962 %F₀/min and 0.173 %F₀/min. Applying Eq. 3 we find that the conservation criterion is not met, indicating that we are not capturing all of the fluorescence in the flanking windows. This is a technical limitation due to the field of view of our EMCCD camera (82 μm x 82 μm). Cameras with sCMOS chips, which can have much larger fields of view, could permit the use of larger flanking window sizes. However, we cannot exclude the possibility that this discrepancy between the central and flanking slopes could also be due, at least in part, to underestimation of the extent of photobleaching (see Discussion), which would have the effect of underestimating the positive slopes in the flanking windows and overestimating the negative slope in the central window.

From the rates in the flanking windows (%F₀/min) and Eq. 2 above, and using values of $v_a = 0.9 \mu\text{m/s}$ and $v_r = 1.2 \mu\text{m/s}$ ¹⁷, we calculate the ratio $p_a/p_r = 2.12$. This indicates that 68% of the filaments were moving anterograde and 32% retrograde. Using the same rates and Eq. 1 above, we calculate the average net population velocity $v = 40 \times (0.00173 - 0.00108) = 0.026 \mu\text{m/min}$, or 0.037 mm/day. Radioisotopic pulse-labeling studies in mice of similar age have reported a neurofilament population velocity of approximately 0.6 mm/day in the most proximal portions of the sciatic nerve, slowing to 0.12 mm/day over a distance of two centimeters^{19,20}. The data was gathered in the tibial nerve, roughly another 2-3 centimeters distal to these measures. For the reasons mentioned above, we believe that the estimate of 0.037 mm/day is an underestimate of the true velocity. However, extrapolating the spatial slowing observed by radioisotopic pulse-labeling into the tibial nerve this estimate is remarkably close given that it was determined using a very different methodology on a very different spatial and temporal scale.

To demonstrate the capability of detecting significant differences between populations using the pulse-spread method, we compared the proximal and distal flanking window slopes measured from nerves perfused with both normal and inhibitor salines. Inhibition of glycolysis blocks movement of neurofilaments, as we have reported previously^{5,6,7}. We will discuss later the selection of sample sizes for experiments, however here we used one nerve in normal saline and two in inhibitor saline, due to the limitation of one acquisition field per nerve during inhibition. **Figure 4A** shows an example timelapse from a glycolytically inhibited nerve, demonstrating an apparent reduction in transport out of the activated region. Indeed, we find significantly lower distal and proximal slopes (**Figure 4B**, $p = 0.00000160$ and 0.00607 , respectively, Tukey's pairwise comparison following ANOVA) in nerves treated with inhibitor versus those perfused with normal saline. We also find a significant decrease in the population velocity between these two conditions (**Figure 4C**, $p = 0.0232$, t-test with unequal variances, following F test for equal variance with $p = 0.0190$).

FIGURE AND TABLE LEGENDS:

Figure 1: The perfusion chamber. (A) Diagram showing the assembly of the perfusion chamber housing and the outer gasket, with tubing connected to the saline syringe and waste flask. (B) Diagram showing the assembly of the chamber itself. The inner gasket is laid flat on top of the #1.5 coverslip. The nerve is placed on the coverslip in the well created by the rectangular opening of the inner gasket. The microaqueduct slide is placed over the nerve to sandwich the nerve between the #1.5 coverslip and the microaqueduct slide. Finally, the sandwich is flipped before mounting on top of the outer gasket of the assembly shown in (A) and secured with a locking ring (not shown).

Figure 2: The tibial nerve preparation. (A) Image showing the location of the tibial nerve within the leg of a mouse, in which the gluteus superficialis, biceps femoris, semitendinosus, popliteus, tibialis caudalis and flexor digitorum longus muscles have been removed. The tibial nerve can be seen as one of three major branches of the sciatic nerve arising at the knee. (B) Schematic of the nerve in cross-section in the assembled chamber, showing the oil-immersion objective beneath the coverslip. The best optical quality is obtained by activating and imaging the layer of axons apposed to the surface of the coverslip; image quality declines deeper into the nerve due to light scattering. (C) Examples of a healthy axon (top), and of an unhealthy axon (bottom) immediately after activation. The dashed yellow line marks the activated region. The activated fluorescence has sharp boundaries in the healthy axon whereas in the unhealthy axon the activated fluorescence diffuses rapidly out of the activated region, filling the axon within seconds. Scale bar = 10 μm . (D) Examples of the mitochondrial appearance in a healthy axon (top) and an unhealthy axon (bottom). The mitochondria are visible due to flavin autofluorescence¹⁶. They appear linear (solid arrowheads) in healthy axons. In unhealthy axons, the mitochondria first become punctate (open arrowheads) and then subsequently fainter over time, providing an indicator of axon health. The dashed open arrowhead in the healthy axon points to a mitochondrion transitioning from linear to punctate. Scale bar = 10 μm .

Figure 3: Pulse-escape and pulse-spread experiments. Example of pre-activation, post-activation and timelapse images of myelinated axons in the tibial nerve of an 8 week old mouse in (A) a pulse-escape experiment and (B) a pulse-spread experiment. Times pre- and post-activation are shown. These activations were performed using 5 scans with a 40 μs pixel dwell time and 5% laser power. Scale bars = 10 μm . (C) The measurement ROIs (yellow) for three axons in a pulse-escape experiment. (D) The proximal (red), central (yellow) and distal (green) measurement ROIs for three axons in a pulse-spread experiment. The measurement ROIs for each axon are shown in solid red (proximal), solid yellow (central window) and solid green (distal window). (E) Plot of the average fluorescence in the three ROIs in C versus time. Dashed line is an exponential function fit to the data, of the form $F_t = Ae^{-\tau t}$, as described previously⁷. (F) Plots of the fluorescence in the central, distal and proximal ROIs versus time, average of 14 axons. The slope is calculated from trendlines fit to the first 5 minutes of data.

Figure 4: The effect of metabolic inhibitors on neurofilament transport. (A) Example of timelapse images of a nerve pretreated with 5.6% (w/v) 2-deoxy-glucose and 0.5 mM sodium iodoacetate to inhibit glycolysis and deplete cellular ATP. Note that the distal and proximal

boundaries of the activated regions remain sharp, indicative of an inhibition of neurofilament transport. Scale bar = 10 μ m. **(B)** Quantification of distal (D) and proximal (P) neurofilament transport rates (anterograde and retrograde, respectively), expressed as percent of initial fluorescence in the central window ($\%F_0/\text{min}$), from one nerve (14 axons) using standard saline and from two nerves (8 axons) pretreated with saline containing the glycolytic inhibitors. **(C)** Neurofilament population velocities calculated from the directional transport rates, showing a significant decrease in velocity following inhibitor treatment. *** - $p < 0.005$; ** - $p < 0.01$; * - $p < 0.05$. The data show significant impairment of neurofilament transport in the presence of the inhibitors.

DISCUSSION:

Care must be taken in the analysis of pulse-escape and pulse-spread experiments because there is significant potential for the introduction of error during the post-processing, principally during the flat-field correction, image alignment and bleach correction. Flat-field correction is necessary to correct for non-uniformity in the illumination, which results in a fall-off in intensity across the field of view from center to periphery. The extent of non-uniformity is wavelength-dependent and thus, should always be performed at the wavelength that is to be used for acquiring the experimental data. It is important to ensure that the non-uniformity in the flat-field image is truly representative of the non-uniformity in the images to be corrected. Pulse-spread experiments are particularly vulnerable to error from improper flat-field correction because the measurement ROIs extend towards the periphery of the image where the intensity fall-off is greatest.

The optimal paGFP activation settings will vary by activation method and laser parameters, and must be determined empirically before the first imaging session. Too little activation will result in a low signal-to-noise ratio for the activated fluorescence, whereas too much will result in bleaching of the activated fluorescence because both the non-activated and activated paGFP can be excited by violet light. To selectively illuminate a region of interest in the image, we use an Andor FRAPPA laser galvo scanner, which generates a clearly defined region of fluorescence with sharp boundaries, as shown in the Representative Results. We have also had success using an Andor Mosaic Digital Diaphragm, which is a digital micromirror device, with a mercury arc lamp as the illumination source⁷. Other options are commercially available. A challenge when working with paGFP is the delayed increase in fluorescence intensity that happens within 1 minute after the photoactivation step. This increase occurs because illumination with violet light causes a proportion of the activated paGFP molecules to enter a “dark state” from which they can relax back on a time course of tens of seconds¹¹. In our experience, the increase is usually less than 5% with widefield excitation but can exceed 20% with laser excitation, which can lead to a significant underestimation of the activated fluorescence. We account for this by acquiring a second “post-activation” image of the paGFP fluorescence 1 minute after photoactivation, and then using this image as the reference point for the subsequent timelapse. However, it is important to recognize that this does introduce another potential source of error in determining the initial fluorescence and the decay kinetics at early times.

Additional attention must be paid to the alignment of the image stacks. The principal source of misalignment is drift or other movement of the specimen during timelapse image acquisition. This can be minimized by using inner gaskets of the appropriate thickness and allowing the preparation to “settle in” before imaging. However, any such delay will reduce the time available for image acquisition since the preparation has a limited window of viability. It is also important to avoid alignment algorithms that warp the image or introduce sub-pixel shifts, as these procedures would resample the pixel intensities in the image. Pulse-spread experiments are particularly vulnerable to errors arising from improper alignment because the borders of the region of activated fluorescence are relatively sharp, and fluorescence in this region is significantly higher than the flanking unactivated regions. If an image is shifted such that a flanking measurement window overlaps the central activated region then the fluorescence intensity in that window will be overestimated. Additionally, the fluorescence increase in flanking windows is typically slow, on the order of 0.05-0.5% of the activated fluorescence in the central window per minute, so small alignment differences between images can result in large jumps in fluorescence values in the distal and proximal measurement windows. Thus, it is imperative that image sets are aligned properly and inspected carefully before proceeding with the analysis.

Correction for photobleaching is necessary to ensure that changes in fluorescence intensity over time accurately reflect changes in the amount of the fluorescent protein. Such corrections can be a significant source of error in estimating the absolute fluorescence intensities in the central and flanking windows. As photobleaching kinetics depend on the intensity of illumination and the environment of the fluorophore, actual photobleaching rates can vary between sessions and from axon to axon. Thus, it is necessary to measure multiple axons and average the resulting data, which itself introduces some error. The approach described above is to treat the nerve with glycolytic inhibitors and then activate the fluorescence in a region of interest and track the loss of fluorescence intensity over time. The glycolytic inhibitors deplete ATP and thus inhibit neurofilament transport so that the loss of fluorescence is due entirely to photobleaching and not movement of neurofilaments out of the activated region. The average bleaching kinetics determined in this way are then used to correct the experimental data. Since it is not practical to perform a separate bleaching calibration in each imaging session, a single calibration must be applied to multiple sessions spread over many weeks. A disadvantage of this approach is that it does not account for variations in laser power/illumination intensity from day to day, which should therefore be monitored. An alternative approach, which we refer to as “intrinsic bleaching correction”, is to estimate the photobleaching by quantifying the fluorescence in the center of the activated region in the same timelapse movies that are used for the transport measurements. If this measurement region is centrally located and much shorter than the length of the activated region, and then at short times any fluorescent neurofilaments that move out of the measurement region will be replaced by fluorescent neurofilaments that move into it. However, with time the probability of non-fluorescent neurofilaments moving into the measurement region from flanking non-fluorescent regions of the axon increases, leading to an overestimation of the loss of fluorescence due to bleaching. An advantage of this approach is that it corrects for the bleaching characteristics within that same field, but a disadvantage is that the duration of the time window must be determined

empirically and will depend on the rate of the transport as well as the length of the activated region. All this having been said, it should be noted that the estimation of the directionality of neurofilament transport using our method is robust to bleaching errors (as it is for flanking window size) because it is given by the ratio of the slopes in the flanking windows and any bleaching correction is a multiplier applied to both numerator and denominator in that calculation.

Due to the noise inherent in a fluorescent system and the potential for additional error added during the image post-processing steps described above, it is important to use large sample sizes for sufficient statistical power. While it is not possible to accurately determine population means, deviations and effect sizes before experimentation, we recommend using the Cohen method²¹ assuming a medium effect size and an alpha of 0.05. This results in a Cohen's *d*, which is the difference in means divided by the pooled standard deviation of both populations, of 0.5, which suggests acquiring at least 105 samples per group. Once this threshold has been reached, one may perform a post hoc power analysis to reassess statistical power in light of actual population measures. Under optimal experimental conditions, it should be possible to obtain from 1-7 analyzable axons (out of 9-20 total axons) per activation, and 5-8 activations per nerve assuming the acquisition of a 10-minute timelapse per activation.

Transgenic mice expressing fluorescent fusion proteins targeted to mitochondria or vesicles have also been used to study axonal transport of membranous organelles in peripheral nerves *ex vivo*²²⁻²⁵. In addition, the Schiavo lab has developed approaches to image the axonal transport of retrogradely moving membranous organelles in axons *in vivo* using fluorescently tagged tetanus toxin fragments, which can be injected into the muscle and are taken up by motor nerve terminals²⁶. However, since membranous organelles can be resolved in these axons by fluorescence microscopy, it is possible to analyze the velocity and frequency of their movement directly using timelapse or kymograph analysis. The pulse-escape and pulse-spread methods described here were developed with the specific goal of analyzing the kinetics of neurofilament transport in these axons, in which single neurofilaments cannot be resolved. This requires a population-level analysis over a period of minutes or tens of minutes. We use a transgenic mouse for this purpose, but it should also be possible to express the photoactivatable protein using methods such as viral transduction or *in utero* electroporation. While the focus has been neurofilament transport, the pulse-escape and pulse-spread methods may also be adapted to study the movement of other cytoskeletal and cytosolic proteins that are transported along axons in the slow components of axonal transport. We also confined the analyses to myelinated axons because their size and myelin sheath allows us to resolve them from their neighbors. Unmyelinated axons cannot be resolved due to their small caliber and tendency to cluster in Remak bundles. We describe the use of tibial nerves *ex vivo* but the methods should also be applicable to other peripheral nerves that are sufficiently long (>5 mm) and unbranched. In principle, it should also be possible to adapt these methods to image neurofilament transport *in vivo* (e.g., by surgically exposing a nerve in a sedated mouse and then placing the mouse on a microscope stage).

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DISCLOSURES:

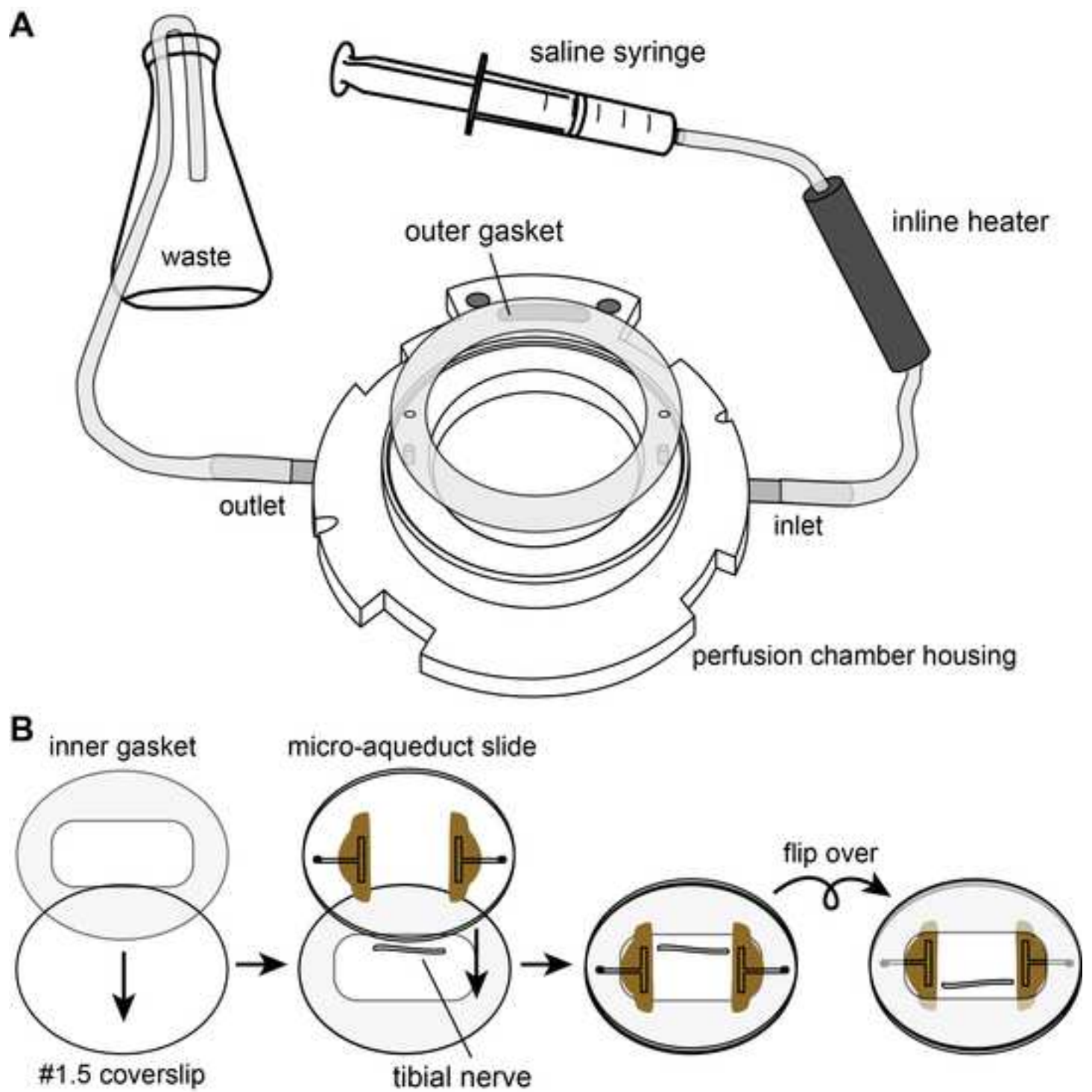
The authors have nothing to disclose.

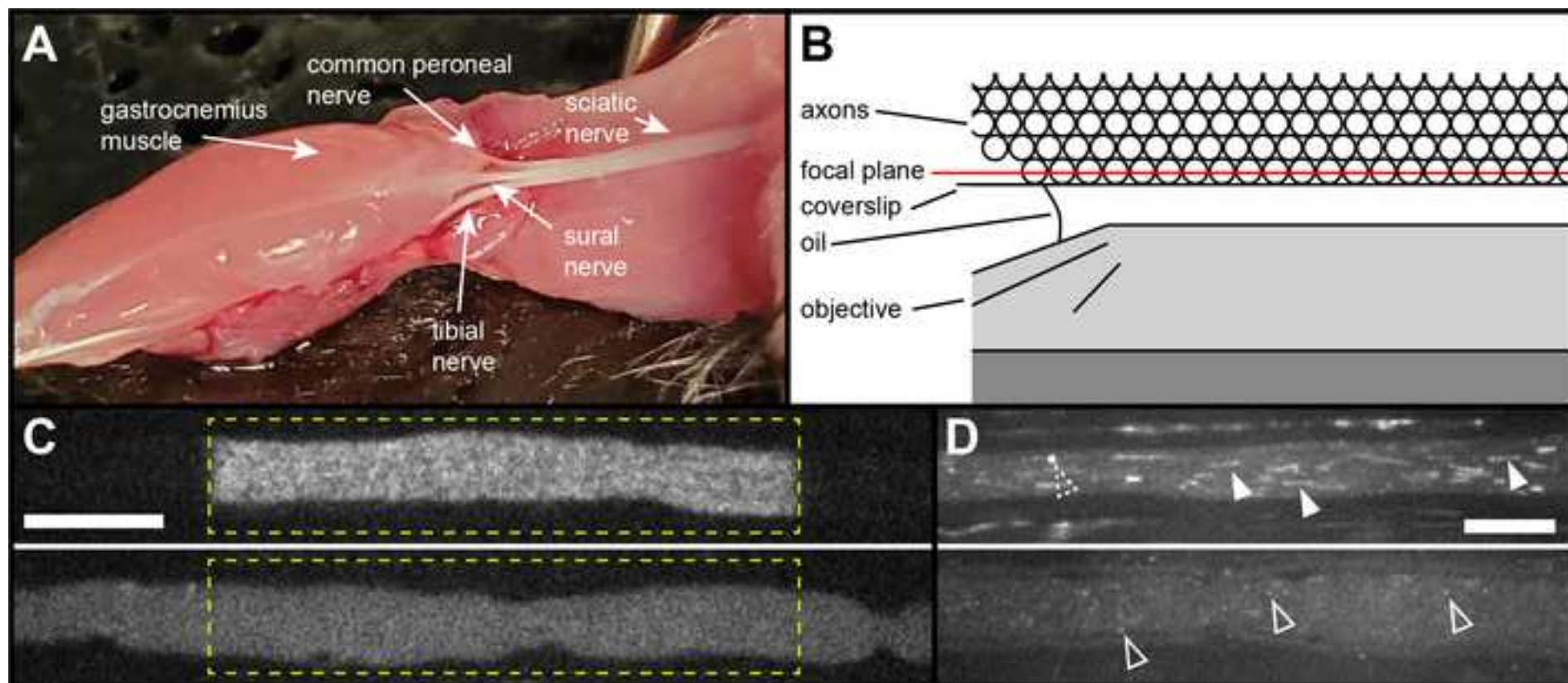
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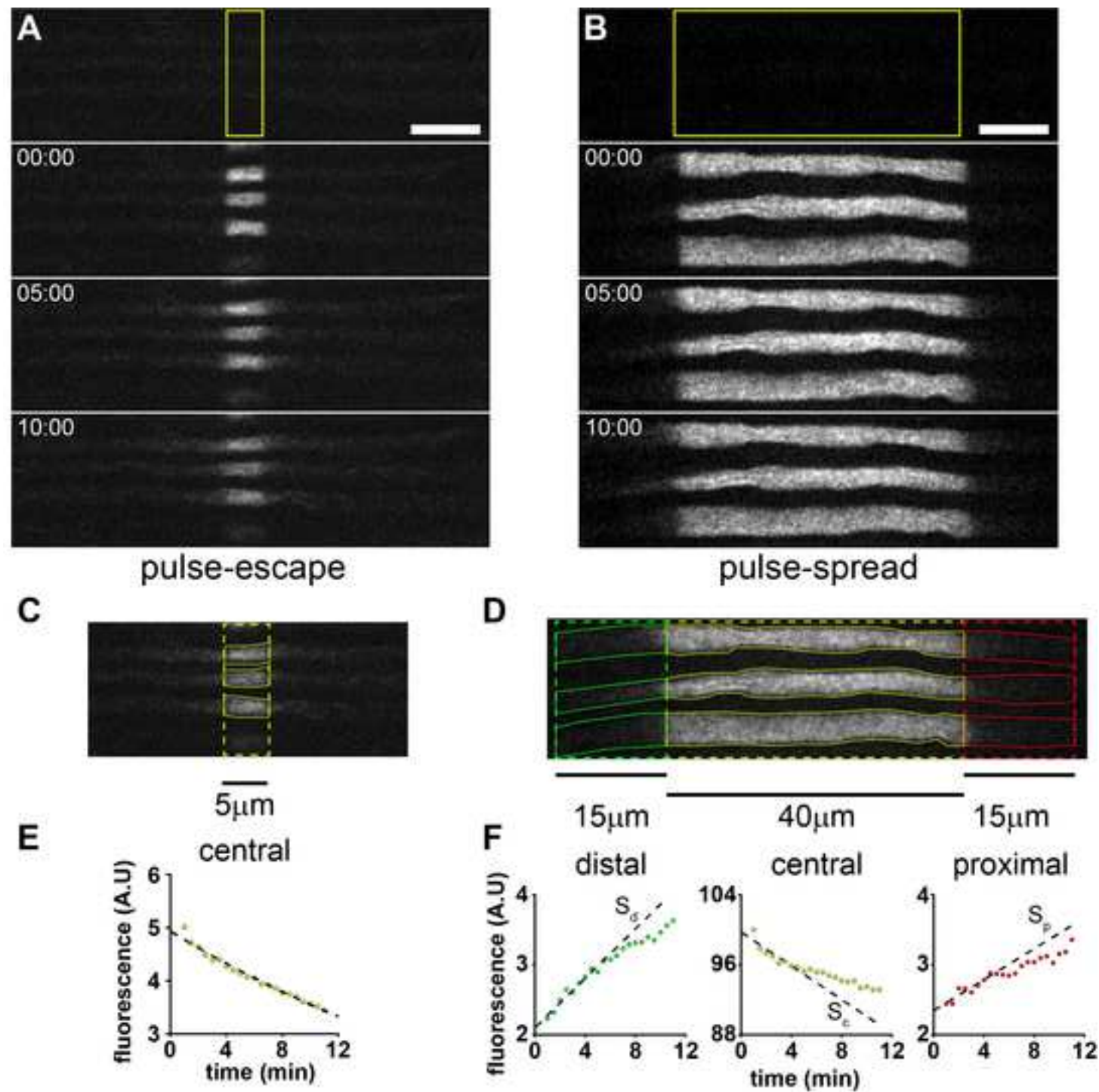
1. Hoffman, P.N., Lasek, R.J. The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurons. *Journal of Cell Biology*. **66**, (2), 351-66 (1975).
2. Brown, A. Slow Axonal Transport. *Reference Module in Biomedical Sciences*. doi:10.1016/B978-0-12-801238-3.04765-6 (2014).
3. Wang, L., Ho, C-L., Sun, D., Liem, R.K.H., Brown, A. Rapid movement of axonal neurofilaments interrupted by prolonged pauses. *Nature Cell Biology*. **2**, (3), 137-141 (2000).
4. Uchida, A., Monsma, P.C., Fenn, J.D., Brown, A. Live-cell imaging of neurofilament transport in cultured neurons. *Methods in Cell Biology*. **131**, 21-90 (2016).
5. Trivedi, N., Jung, P., Brown, A. Neurofilaments switch between distinct mobile and stationary states during their transport along axons. *Journal of Neuroscience*. **27**, (3), 507-16 (2007).
6. Monsma, P.C., Li, Y., Fenn, J.D., Jung, P., Brown, A. Local regulation of neurofilament transport by myelinating cells. *Journal of Neuroscience*. **34**, (8), 2979-2988 (2014).
7. Walker, C.L. et al. Local Acceleration of Neurofilament Transport at Nodes of Ranvier. *Journal of Neuroscience*. **39**, (4), 663-677 (2019).
8. Li, Y., Brown, A., Jung, P. Deciphering the axonal transport kinetics of neurofilaments using the fluorescence photoactivation pulse-escape method. *Physical Biology*. **11**, (2), 026001 (2014).
9. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nature Methods*. **9**, 676-682 (2012).

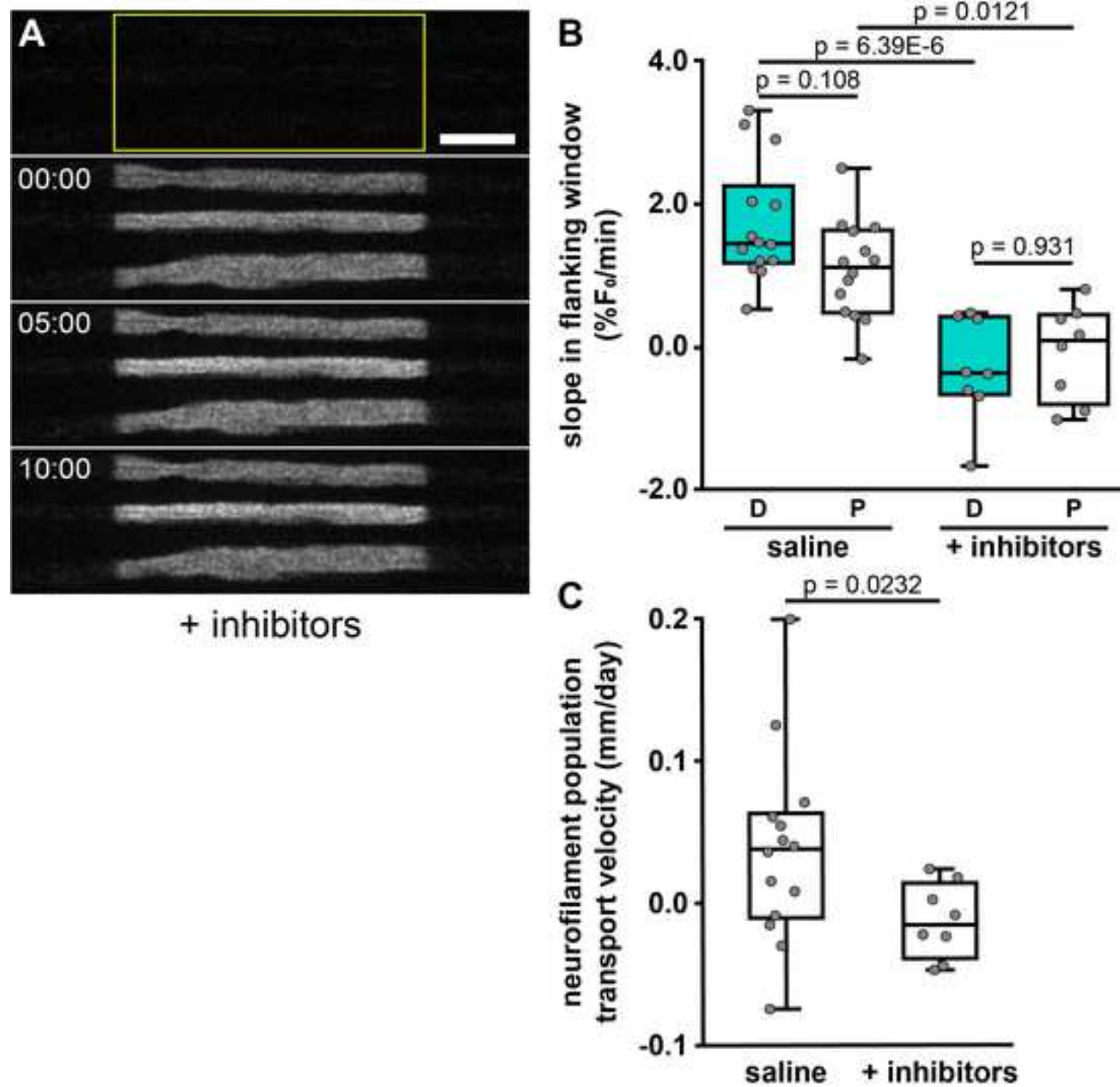
10. Breuer, A.C. et al. Fast axonal transport in amyotrophic lateral sclerosis: an intra-axonal organelle traffic analysis. *Neurology*. **37**, (5), 738-48 (1987).
11. Bancaud, A., Huet, S., Rabut, G., Ellenberg, J. Fluorescence perturbation techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, photoactivation, photoconversion, and FLIP. *Cold Spring Harbor Protocols*. **2010**, (12) (2010).
12. Model, M. Intensity calibration and flat-field correction for fluorescence microscopes. *Current Protocols in Cytometry*. **68** (2014).
13. Schmidt, M.M., Dringen, R. Differential effects of iodoacetamide and iodoacetate on glycolysis and glutathione metabolism of cultured astrocytes. *Frontiers in Neuroenergetics*. **1**, 1-10 (2009).
14. Surre, J. et al. Strong increase in the autofluorescence of cells signals struggle for survival. *Scientific Reports*. **8**, (1), 12088 (2018).
15. Cox, M., Lucey, S., Sridharan, S., Cohn, J. Least Squares Congealing for Unsupervised Alignment of Images. *Proceedings of the IEEE Computer Society Conference on Computer Vision and Pattern Recognition*. doi:10.1109/CVPR.2008.4587573 (2008).
16. Huang, S., Heikal, A.A., Webb, W.W. Two-photon fluorescence spectroscopy and microscopy of NAD(P)H and flavoprotein. *Biophysical Journal*. **82**, (5), 2811-25 (2002).
17. Fenn, J.D., Johnson, C.M., Peng, J., Jung, P., Brown, A. Kymograph analysis with high temporal resolution reveals new features of neurofilament transport kinetics. *Cytoskeleton*. **75**, (1), 22-41 (2018).
18. Alami, N.H., Jung, P., Brown, A. Myosin Va increases the efficiency of neurofilament transport by decreasing the duration of long-term pauses. *Journal of Neuroscience*. **29**, (20), 6625-6634 (2009).
19. Xu, Z., Tung, V.W. Temporal and Spatial Variations in Slow Axonal Transport Velocity Along Peripheral Motoneuron Axons. *Neuroscience*. **102**, (1), 193-200 (2001).
20. Jung, P., Brown, A. Modeling the Slowing of Neurofilament Transport Along the Mouse Sciatic Nerve. *Physical Biology*. **6**, (4), 046002 (2009).
21. Cohen, J. The effect size index: d. *Statistical Power Analysis for the Behavioral Sciences*. Lawrence Erlbaum Associates. **2nd ed.**, 20-26 (1988).
22. Misgeld, T., Kerschensteiner, M., Bareyre, F.M., Burgess, R.W., Lichtman, J.W. Imaging axonal transport of mitochondria in vivo. *Nature Methods*. **4**, (7), 559-61 (2007).

23. Gilley, J. et al. Age-dependent axonal transport and locomotor changes and tau hypophosphorylation in a "P301L" tau knockin mouse. *Neurobiology of Aging*. **33**, (3), 621.e1-621.e15 (2012).
24. Marinkovic, P. et al. Axonal transport deficits and degeneration can evolve independently in mouse models of amyotrophic lateral sclerosis. *Proceedings of the National Academy of Science of the United States of America*. **109**, (11), 4296-301 (2012).
25. Milde, S., Adalbert, R., Elaman, M.H., Coleman, M.P. Axonal transport declines with age in two distinct phases separated by a period of relative stability. *Neurobiology of Aging*. **36**, (2), 971-81 (2015).
26. Gibbs, K.L., Kalmar, B., Sleight, J.N., Greensmith, L., Schiavo, G. In vivo imaging of axonal transport in murine motor and sensory neurons. *Journal of Neuroscience Methods*. **257**, 26-33 (2016).









Name of Material/ Equipment	Company	Catalog Number
14 x 22 Rectangle Gasket 0.1mm	Bioptechs	1907-1422-100
2-deoxy-D-glucose	Sigma	D6134
30mm Round Gasket w/ Holes	Bioptechs	1907-08-750
35 x 10mm dish	Thermo Fisher	153066
40mm round coverslips	Bioptechs	40-1313-0319
60mL syringe - Luer-lock tip	BD	309653
Andor Revolution WD spinning-disk confocal system	Andor	
Calcium chloride	Fisher	C79
Coverslips	Fisher	12-541-B
D-(+)-glucose solution	Sigma	G8769
Dissecting pins	Fine Science Tools	26001-70
Dissection forceps	Fine Science Tools	11251-30
Dissection microscope	Zeiss	47 50 03
Dissection pan with wax	Ginsberg Scientific	568859
Dissection scissors	Fine Science Tools	14061-09
FCS2 perfusion chamber	Bioptechs	060319-2-03
Fluorescein sodium	Fluka	46960
Inline solution heater	Warner Instruments	SH27-B
Laminectomy forceps	Fine Science Tools	11223-20
Magnesium sulfate	Sigma-Aldrich	M7506
Microaqueduct slide	Bioptechs	130119-5

Microscope slides	Fisher	12-544-3
Microscope stage insert	Applied Scientific Instrumentation	I-3017
Objective heater system	Okolab	
Objective oil - type A	Nikon	discontinued
Plan Apo VC 100x 1.40 NA objective	Nikon	MRD01901
Potassium chloride	Fisher	P217
Potassium phosphate	Sigma-Aldrich	P0662
Sodium bicarbonate	Sigma-Aldrich	S6297
Sodium chloride	Sigma-Aldrich	S7653
Sodium iodoacetate	Sigma-Aldrich	I2512
Syringe pump	Sage Instruments	Model 355
Tubing adapter - female	Small Parts Inc.	1005109
Tubing adapter - male	Small Parts Inc.	1005012
Tygon tubing	Bioprotechs	
Vannas spring scissors	Fine Science Tools	15018-10

Comments/Description

inner gasket

outer gasket

dissection dishes

outfitted with Perfect Focus and
FRAPPA systems

for fluorescein slide

fine tipped forceps

initial dissection scissors

initial dissection forceps

for fluorescein slide

Oko Touch with objective collar

1/16" ID, 1/32" wall thickness

fine scissors

We thank the editor and reviewers for their detailed comments and suggestions, which have helped us improve the manuscript significantly. Please find below a detailed response to all of the reviewers' comments. The reviewers' comments are shown in black font and our responses are shown in blue font.

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please revise lines 65-67, to avoid textual overlap with previously published work.

We are unclear on the overlap between this sentence and previous work, however we have rephrased it to now read:

"To analyze the axonal transport of neurofilaments in neurofilament-rich axons using fluorescence microscopy, we use a fluorescence photoactivation pulse-escape method that we developed to study the long-term pausing behavior of neurofilaments in cultured nerve cells"

3. Authors and affiliations: Please provide an email address for each author in the manuscript.

We have added email addresses for the non-corresponding authors.

4. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE."

All instances of the phrases "could be", "should be" and "would be" have been replaced with wording more appropriate to authoritative instructions.

5. In the JoVE Protocol format, "NOTE" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Several of the notes have been removed, others have been shortened and rephrased, and some have been moved to the discussion section. The remaining notes are those parts of the protocol which are critical information for the understanding of each step yet are not imperative directions.

6. 3.1: Please specify the age, gender and strain of mouse used here. Please also specify the euthanasia method used.

We have added information on the sex and strain of the mouse used in the representative results alongside the age, and have added the following sentence:

"Mice of at least 2 weeks of age, both male and female, have been used for this experimental paradigm, and the appropriate age and sex of mice should be determined by the researcher depending on what is being tested in the study."

The euthanasia method used here has also been included in step 3.1.

7. 5.4: Please specify the excitation and emission filters used.

We have added details on the emission filter (525/50 nm) and removed reference to the excitation filter. In this protocol, a 488nm laser is used with no additional filter on the excitation side.

8. 5.6: Please specify the settings.

The wording has been changed to clarify that the settings to be used in 5.6 are those used in 5.4.

9. Figures: Please include a space between the number and the units of the scale bar.

We have added a space between the number and units in Figure 1.

10. Table of Materials: Please sort the materials alphabetically by material name.

The Materials table has been sorted appropriately.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

How it imagine NF transport in vivo (sic).

Major Concerns:

None

Minor Concerns:

None

Recommendation:

I strongly support this. The Brown group at Ohio State is the only one capable of doing this kind of thing and it's essential that others can too.

Reviewer #2:

Manuscript Summary:

In the manuscript "Imaging and Analysis of Neurofilament Transport in Excised Mouse Tibial Nerve", Boyer et al. present a fluorescence photoactivation-based method for determining neurofilament transport rates in mouse peripheral nerves. This is a useful technique, and should be of interest to anyone working on axonal transport processes. In general, the protocol is laid out very clearly, with enough detail given to ensure reproducibility by readers, and with all required controls and corrections included. This method involves several steps (e.g. nerve dissection and prep, chamber setup, imaging setup) that are difficult to communicate in standard written protocols, and should thus be a good candidate for publication as a JOVE video.

Manuscript comments:

1) It is somewhat misleading to refer to the technique as "in vivo" (e.g. in the abstract) as clearly the animal is dead and the tissue removed from the animal by the time the experiment occurs. "Ex vivo" is the more appropriate term and the authors do use this term in the manuscript - but this should be consistent throughout.

We do agree that the term "*in vivo*" was used somewhat inappropriately, and have replaced this phrase with *in situ* in several places. Our intent here is to convey that both the radiolabeling assays used previously, and the pulse-spread/escape approaches described here, measure the movement of neurofilaments in axons as they are in the animal, as opposed to *in vitro*. The phrases *in vivo*, *ex vivo*, and *in situ*, are now used more consistently.

2) The instructions for assembly of the perfusion chamber as well as the nerve prep are difficult to follow based on the text and Figure 1 only. I expect the video will be much easier to understand in this regard. However, if the text is to stand alone as an instruction/protocol, I would suggest that some intermediate snapshots from the assembly/nerve prep procedure are included as an additional figure.

We have added extra panels to Figure 1 which should make the assembly and preparation easier to understand. The additional images, along with the video, should make the assembly clear.

3) Step 5.5 - Could the authors include data to show that this step does indeed improve signal-to-noise and, more importantly, does not cause photo-toxicity due to the prolonged illumination with high laser power?

We have revised our language to clarify that this step is not essential, but we have found it to be beneficial. To show data to support this would add another figure and we already have 4 figures. While it is impossible to rule out any phototoxicity associated with fluorescence imaging, we should note that even at 5x laser power (25% on our system), the intensity is still very low because we are using spinning disk confocal microscopy which spreads the light that would normally be focused into one spot across 20,000

spots. Moreover, using the morphological criteria that we describe (mitochondrial fluorescence and morphology, in particular) we do not see any evidence of toxicity associated with this bleaching step.

4) Step 5.7/5.8 - Some additional clarification is required on how to ensure that the size of the ROI is exactly the same from experiment to experiment. Most microscope systems allow users to save ROI parameters to help with this. (note the authors actually do this in step 5.11, but I think a reference to this would be useful at this point in the protocol)

The exact width and orientation of the activation ROI will vary from field to field because the number of axons being activated and their angle with respect to the camera will vary. However, the length of the activation ROI in the axial dimension of the axons is kept constant by using the measurement line as a guide, as described in step 5.7/5.8. The video should clarify how this maintains a fixed window length.

5) Step 5.9 - Clearly getting the PA settings right is crucial to the success of the entire experiment. I feel that the guidance given by the authors on how to optimise PA settings is too general. Could they perhaps suggest a more step-by-step approach to setting up PA? E.g. would a sequence of laser pulses of increasing power be a good way to find optimum settings? Is exposure time or laser power more important in determining PA efficiency and photo-bleaching? Which should be optimised first? Which tools could be used to verify the PA setup is working on a given system before commencing animal work?

This is a helpful suggestion. We have added a series of steps (5.9.1-5.9.4) detailing how photoactivation settings can be determined, assuming that the reader uses a similar photoactivation system. Adjusting either laser power or exposure time (or in the case of these steps, number of exposures) will increase the total photon load on the activation area, however as laser power adjustment is often nonlinear with respect to output, exposure time allows finer control. Pulse number is used here as it allows a simple quantization of exposure increase (e.g. 1, 2, 3 pulses), as well as a brief time between pulses (in the case of the FRAPPA galvo-scanning system, the time to scan all other pixels) which reduces photobleaching (T. Bernas, M. Zarębski, R.R. Cook, J.W. Dobrucki. Minimizing photobleaching during confocal microscopy of fluorescent probes bound to chromatin: role of anoxia and photon flux. *Journal of Microscopy*. v.215(3). 2004.). No tool is suggested for the PA optimization, as plotting the fluorescence after activation versus exposure time and/or pulse number should be sufficient to tell whether activation is working.

6) Step 5.10 - Do the authors have some data that shows the effect of the 1-minute delay prior to commencement of imaging? They quote figures of 5% and 20% for widefield and confocal systems respectively and it would be good to show where these numbers come from.

Data on this is found in the reference that we cite in that step of the protocol (Bancaud, et al. 2010).

7) Step 5.12 - How many axons / fields of view should be acquired from a single nerve?

We have added a sentence to the penultimate paragraph of the Discussion to address this. The number of myelinated axons obtained per field of view will vary widely depending on the age of the animal and the location along the nerve (axon diameter and therefore packing changes with age and distance along the nerve), as well as other anatomical features of a given field (e.g. number of Remak bundles). A single field of view contains 9-20 axons, or which about 1-6 are typically analyzable. The number of fields of view that can be analyzed in one nerve in one imaging session will depend on the amount of time taken to extract the nerve, assemble the perfusion chamber and prepare the microscope, as well as the amount of time taken to find each suitable field of view (axons are in focus for the full width of the field and linear) and length of timelapse. The only limitation to the number of fields acquired is the 3 hour postmortem window. With proficient dissection, we are typically able to acquire 10-minute timelapse image series in 5-8 fields of view for each nerve.

8) Step 5.12 - Are there changes during the first 3 hours - i.e. is there data the authors could show to demonstrate that the transport kinetics do not change during the first 3 hours after dissection? From our own experience (admittedly with fast axonal transport cargoes and a slightly different setup), transport rates in sciatic and tibial nerve start to slow down after one hour post-dissection. So if the author's preparation produces stable transport rates for 3 hours, it would be interesting to see that data explicitly. In the cited Walker et al. (2019) paper, data is shown on mitochondrial transport rates using this experimental setup. We did not observe slowing of mitochondrial transport over the course of the first

three hours. We have not investigated whether neurofilament transport specifically changes during these 3 hours, but the mitochondrial motility data demonstrate that the preparations are viable.

9) Step 7 - It is not clear at this point whether the authors intend for readers to carry out bleach correction experiments on each experimental day or whether a general reference set is used. This should be clarified. Arguably, if imaging and activation settings are kept constant between sessions, the bleach reference could be re-used. Aside from the additional time and cost, this is mostly a question of whether the use of additional animals on each experimental day is justified and required for this purpose. It would be interesting to see data on the day-to-day variation in bleach correction images if the authors do indeed capture these on each occasion.

We do indeed use the same control data for a given set of experiments across many imaging sessions, as we have found that our laser power remains fairly consistent over time and all acquisition and activation settings are kept constant in all sessions. To clarify this and address the reasoning for it, we have added the following to the Discussion:

"The average bleaching kinetics determined in this way are then used to correct the experimental data. Since it is not practical to perform a separate bleaching calibration in each imaging session, a single calibration must be applied to multiple sessions spread over many weeks. A disadvantage of this approach is that it does not account for variations in laser power/illumination intensity from day to day, which should therefore be monitored."

Perhaps the authors could also comment on other possible ways to obtain bleach correction images that do not require additional nerves prepped in inhibitory saline. One straightforward way would be to photoactivate an entire field of view, carry out the time lapse imaging as usual, and then quantify the decline in fluorescence in a small central ROI of the image. Even though transport is still carrying on under these conditions, the length of time for which the axons are imaged/analysed for (i.e. 5-10 min) would not be long enough for unlabelled material to reach the centre of the field of view. Everything being transported in and out of the small central ROI would be similarly exposed to photobleaching during the entire experiment, with a net-zero effect on transport. Any decline in fluorescence would be solely due to photobleaching.

This is an excellent suggestion by the reviewer, and in fact this "intrinsic correction" was our original method of photobleach correction. We have included it in the discussion as an alternative method, however it can overestimate the bleach rate substantially when the neurofilament transport rate is fast because in this case some of the loss of fluorescence is due to transport not photobleaching. The data presented in this manuscript are from a population in which intrinsic bleach correction is frequently appropriate, however in some of our yet-to-be-published data the transport rate is sufficiently high to preclude intrinsic correction.

10) Based on the lack of yellow highlighting, it seems that the entire image and data processing workflow will not be shown in the video. As there are quite a few processing steps, and as these are critical for creating a meaningful output, this section is crucial. In my opinion a series of screenshots would make this easier to follow, either as part of the video (preferably) or as an additional figure. For example, the alignment of the ROIs described in step 8.3.5 and 8.3.6 is somewhat difficult to understand without visual guidance.

We excluded the analysis steps because of the 2.75-page limit on the number of protocol steps that can be shown in the video. We will ask the editors whether it would be possible to include more steps in the video, as we agree that it would be helpful to show the analysis process.

11) The calculations presented in the Representative Results section seem to assume that individual neurofilament polymers do not change direction. Can the authors demonstrate that this is a valid assumption for the timescales of the experiment? If not, would it not be necessary to include additional terms into, for example, the equations on line 523 to account for the fact that polymers could return from the flanking windows to the central region? This would then affect the estimates of average velocities (line 548).

We do not make this assumption. When the center window is activated all neurofilaments in all kinetic states become fluorescent. At short times after activation the only thing that contributes to the fluorescence in the flanking windows are the fractions ϕ_{A} and ϕ_{B} of neurofilaments in the anterograde and

retrograde *motile* states at the time of activation, resulting in a linear increase in the flanking windows. These fractions are determined by the reversal rates γ_{retro} and γ_{ant} which we do not know individually, but whose ratio can be estimated using the slopes \square_{retro} and \square_{ant} . We have revised Eq. 2 to indicate this.

12) Line 590/591: Could the authors comment on how these rates compare to estimates obtained using "traditional" methods such as radiolabelling? Perhaps a short paragraph on this topic could be included in the discussion?

We now address this in the penultimate paragraph of the Representative Results section. A separate experimental paper using the approaches described here is in preparation, and that paper will address this issue more thoroughly, including much larger data sets and in animals of different ages.

13) Line 670/671: Could the authors show/reference data to demonstrate this is indeed the case?

This comment referred to the sentence "As the variation between individual axons is as broad as that between nerves, it is possible to use each axon as a separate sample for statistical analysis." We have removed this line from the discussion, as there isn't enough space within the confines of a JoVE article to include such data, and this analysis requires a larger data set than that which is included in this article.

14) One thing that I feel is missing from the Representative Results section is a demonstration that the novel pulse-spread technique can detect changes to neurofilament transport in different experimental conditions. The authors do comment on the number of axons required to achieve sufficient statistical power; however, it would be helpful to see a dataset where a significant difference in transport rates has been observed between experimental conditions to demonstrate the utility of this method.

To address this, we have included an additional figure (Fig. 4) which includes example images from axons pretreated with deoxyglucose and iodoacetate as well as neurofilament transport rate and velocity measurements from one nerve perfused with normal saline, and two nerves with inhibitor saline. This small data set is sufficient to show significant differences in both transport rate and population velocity between inhibited and uninhibited axons. We are currently preparing a manuscript which includes larger data sets in line with our suggestions on sample size in the Discussion.

Figure 1 - labels are low-resolution and difficult/impossible to read in my copy (especially panel B).

The figures as submitted are 300dpi with all fonts at least 12 points, and as such should be readable. We are unclear as to why the images that the reviewer received were of low resolution.

Reviewer #3:

In this manuscript, the authors present two related methods to measure axonal transport of neurofilaments. The methods are based on the use of transgenic mice that express neurofilament M linked to photoactivatable (pa) GFP. Nerves are isolated from the mice, placed in a perfusion chamber and axons (myelinated large caliber ones) are imaged. The labeled neurofilaments are photoactivated in a restricted section of the axons, and their dispersal is quantified. Approaches to correct for illumination inhomogeneity, bleaching and drift are introduced and discussed.

Pulse-escape refers to a method in which the fluorescence within a short photoactivated segment is followed over time. In pulse-spread, a longer segment is activated; the fluorescence within this segment and in adjacent proximal (retrograde transport) and distal (anterograde transport) segments is quantified. The first method allows calculating an average transport speed, without providing information about differences in anterograde and retrograde transport. The second provides information about transport bias as well.

The technical aspects of the methods are described comprehensibly and in a manner which should allow others to repeat these types of experiments. However, it is my impression that the theoretical description of the analysis is less complete.

Following are my comments on the manuscript and figures (order as in manuscript):

1. Is oxygenation performed by bubbling with 95% oxygen gas, or 95%/5% oxygen/carbon dioxide

(carbogen)? The composition of the saline, which includes sodium bicarbonate implies the use of 5% carbon dioxide, however this is not specifically mentioned. Do gas bubbles sometimes form in the imaging chamber?

The oxygenation indeed uses carbogen, and while this was included in the materials list, it is now explicitly stated in the protocol as well. Additionally, we have included the following text in a later step to account for the occasional formation of bubbles in the perfusion chamber:

“Changes in temperature may cause bubbles to form in the perfusion chamber due to outgassing of the solution. If bubbles form, briefly increase the solution flow rate by 5-10x until bubbles clear the chamber.”

2. In my experience with experiments involving paGFP, it is critical to minimize light exposure during the preparation phase, to avoid inadvertent activation of paGFP prior to the commencement of the experiment. Such activation is very detrimental to the SNR during the experiment. This is true for any environmental light, as well as light used during the surgical procedures. It is probable that using filtered light (to remove violet/blue components) can help in this respect. I do realize that the prebleaching step solves this issue partially, but it is better to minimize it apriori.

We have not specifically explored the potential for incidental paGFP activation during preparation steps, however our SNR (as is now reported in step 5.4) remains quite high and, as noted by the reviewer, our pre-bleaching step should minimize the effect of incidental light exposure. We have added the following to step 3.7 of the protocol and to the discussion to address this possibility:

“Note: In all steps including and following dissection of the tibial nerve, avoid unnecessary exposure to ambient blue and violet light to minimize possible activation of paGFP in the nerve.”

3. It would be helpful to indicate where the nerve sheath should be grasped in 3.11.

We have added the following to the text to clarify this, while the video should clear up any further confusion:

“grasp the sheath proximally and slowly pull towards the distal end of the nerve”

4. The descriptions given by the authors related to laser power etc. are not very helpful. The specific power of the lasers that should be used is significantly dependent on a very large number of factors which will differ between systems: the full power of the laser, the transfer efficiency of the optical components, the numerical aperture of the objective, the specific color filters used etc. It would be more helpful to indicate what is the desired SNR, and how much bleaching is acceptable during an experiment.

We agree. We have included the following sentence in step 5.4 to address this:

“After photoactivation, the ideal imaging settings will produce a signal-to-noise ratio > 8 and photobleaching of less than 25% of the original signal over the course of 20 images.”

5. The authors indicate in 5.5 the necessity to perform a prebleaching step to reduced autofluorescence. As indicated above - autofluorescence is only part of the reason that green fluorescence may be observed at this stage.

The following has been added to step 5.5 to include the possibility of other sources of fluorescence being pre-bleached:

“...and other sources of unwanted fluorescence...”

6. In 6.4-6.5 the authors use different terms to refer to acquired images. “25 fields of view”, “acquire a stack of 100 images”. This is somewhat confusing.

In this context, the phrases “fields of view” and “images” are referring to two different things. Fields of view in step 6.4 refers to acquiring an image, moving the stage, and acquiring another image for a total of 25 different areas. In step 6.5, the 100 images are acquired sequentially without moving the stage. The following language has been added to step 6.4 to accentuate this difference:

“moving the stage by at least 20µm in any direction between images”

7. In 6.5 - “bias offset on the camera chip” or of the confocal PMT.

We mean the camera chip. In the case of spinning disk confocal microscopy the detector is the camera chip. Spinning disk confocal microscopes do not use a PMT.

8. In 8.3.1: add “to the first” after “vertical”.

The term “vertical” is not relative to the other arm of the angle. By vertical, we mean upright in the image as displayed.

9. In 9.1 “each other frame” should be “each frame”.
The word “other” has been removed.

10. The term “tau” is typically used to describe time constants (with units of time). The way the authors wrote the exponential formula, tau is not a time constant, but a rate constant with 1/sec units. The formula should be $A \exp(-t/\tau)$.

The τ in this formula (and other references to this rate) has been replaced with a γ which represents the exponential decay rate.

11. “e” is usually called the natural logarithm base or Euler's number. A natural number is any positive integer. I actually think it is more than sufficient to just write “e” without any additional explanation.

This has been corrected to read “the natural logarithm base”.

12. In my opinion it would make more sense in 9.5 to use actual time in finding the time constant of bleaching, as well as in the correction formula, because if there are any deviations in the timing of acquisition, then the correction scheme that is described by the authors will lead to an error in correction. I realize that if the time intervals are precise, this is not a problem.

We agree. Frame number has been changed to time in both places.

13. The authors should address the possibility that free diffusible paGFP exists in the axons due to breakdown of the labeled neurofilament. It would make sense to examine by any method (for example Western blots) how much of the paGFP may not be attached to a full neurofilament protein. The existence of any quantity of free paGFP can lead to wrong conclusions.

Western blotting with NFM and GFP antibodies shows a single band corresponding to the paGFP-NFM fusion protein (Walker et al, 2019) so we have no evidence for breakdown of the labeled neurofilament, and this is consistent with the known stability of neurofilament proteins in axons. We do observe that the activated fluorescent neurofilament protein is diffusible in unhealthy axons (see Fig. 2C). This is presumably due to calcium-activated proteolysis and disassembly of the neurofilament polymers in axons resulting from a collapse of the axon's membrane potential and an influx of calcium. In contrast, activation of the fluorescence in a healthy axon produces a discrete activated region with sharp boundaries that spread gradually over a period of many minutes (Fig. 3A,B). This spreading can be detected by an increase in fluorescence in the flanking windows in the pulse-spread analysis (Fig. 3F). Treatment with glycolytic inhibitors to deplete ATP blocks this increase, consistent with the paGFP-NFM protein being non-diffusible (Fig. 4). We have added a disclaimer in the Discussion that we cannot exclude the possibility that there is a small fraction of diffusible neurofilament protein. However, it is important to note that any such diffusible protein would exit the activated region within seconds and thus would not contribute to the pulse-spread or pulse-escape kinetics on the timescales that we measure them.

14. In their description of the theory of the analysis of the experimental results, I was missing a more rigorous description of the formulae. For example, what are the units of the various constants/variables? For example, flux is usually defined as the movement of material across a cross section. This is not the definition the authors used. Therefore, they should make it clearer what the units are. Likewise, the authors do not clarify whether they use signs to indicate the direction of movement - in some formulae it appears that the velocities are signed, in others not. If this is not the case, please explain.

As stated in the Representative Results section, we define the flux j to be the quantity of neurofilament polymer that moves past a particular location in a unit of time. If the window size is expressed in μm and the velocity in $\mu\text{m/s}$ then the flux will have units s^{-1} . If the velocity is expressed in $\mu\text{m/s}$, then the slopes must be expressed in s^{-1} . We have added a sentence to the Representative Results to explain this. The flux of material across a cross-section is flux density, but here flux is a one-dimensional quantity (anterograde or retrograde) as it is integrated across the entire cross section of the axon. The anterograde and retrograde velocities are considered positive so they are not signed. The sign is shown separately to make it clear that retrograde movement is negative. For example, the retrograde flux is

expressed as $j_r = -np_r v_r$. We will present a more detailed derivation of these expressions, with a more detailed description of the analysis, in a paper that is in preparation.

15. The differential formulae appear to produce linear functions over time. I do not expect this to be the case. The authors write in the legend of figure 2 that the graphs are fit by exponents (the expected result) yet in the text (for example in line 530), they refer to slopes (of lines). Are these experiments really expected to produce linear plots in relation to fluorescence over time? This is a key issue regarding this review. The authors write that they refer to the initial 5 minutes (line 582), which is consistent with the calculation of an initial rate. What is the rest of the time lapse used for?

We have shown previously (Li et al., 2014) that the pulse-escape kinetics at later times can be used to constrain estimates of the long-term pausing behavior of the neurofilaments. However, for the pulse-spread paradigm we use only the kinetics at short times. With both methods, the decay kinetics are well fitted by a double exponential function with a slower decay at later times. However, we showed in Li et al (2014) that the decay is strictly linear at very early times during which motile neurofilaments (neurofilaments that are on track and moving at the time of photoactivation) leave the center window. The transition between the linear and exponential regimes, however, is smooth, especially for the large window sizes used here (see Eq.25 of Ref.8), resulting in an exponential decay which follows a linear approximation for the first 5 minutes or more (see e.g. Fig.5 in Ref.8 for a window size of $50 \mu\text{m}$). Note that the Taylor expansion of an exponential function is linear in leading order, i.e. $\exp(-x) = 1 - x + \frac{x^2}{2} - \dots$. Consistently, the fluorescence increase in the flanking windows in the pulse-spread method is also approximately linear for a similar time period, and the duration of this period also increases with increasing window length. The derivation of the equations describing the linearity of the fluorescence increase at short times is beyond the scope of this protocol, but will be described in detail in a paper that we are currently preparing. Thus, there is a sound theoretical basis for the linearity of the slopes in the central and flanking windows at short times.

16. The sign of J_r in line 510 and 540 is not consistent.

We apologize for this oversight and have corrected the sign of j_r in the formula in line 540 (now $-j_r/na$).

17. In line 552, can one really claim that the retrograde and anterograde speeds are similar enough to assume their ratio is 1?

We thank the Reviewer for drawing our attention to this. It is not necessary to make this approximation so we have removed it. The expression now reads $p_a/p_r = (s_d/s_p)(v_r/v_a)$. We have measured v_a and v_r in cultured neurons to be 0.9 and 1.1 $\mu\text{m/s}$ respectively (Fenn et al, 2018), yielding a ratio $v_r/v_a = 1.22$. We have added the following sentence:

"The values of v_r and v_a can be determined by measuring the movement of individual neurofilaments in cultured neurons, as reported previously (Fenn, 2018)."

18. In 585 - the rates described for the central and flanking sections do not sum up. Are they area-corrected? Was there loss of fluorescence?

For the data shown in Fig. 3, the Reviewer is correct that the sum of the slopes in the flanking windows does not equal the negative slope in the central window. This is at least partly because the flanking windows were not long enough to capture all of the fluorescence entering from the center window, but it may also be due to underestimation of the extent of photobleaching (see Discussion). We have added the following explanation to the Representative Results: "Applying Eq. 3 we find that our conservation criterion is not met, indicating that we are not capturing all of the fluorescence in the flanking windows. This is a technical limitation due to the field of view of our EMCCD camera ($82 \times 82 \mu\text{m}$). Cameras with sCMOS chips, which can have much larger fields of view, could permit the use of larger flanking window sizes. However, we cannot exclude the possibility that this discrepancy between the central and flanking slopes could also be due, at least in part, to underestimation of the extent of photobleaching (see Discussion), which would have the effect of underestimating the positive slopes in the flanking windows and overestimating the negative slope in the central window." Additionally, we have added the following sentence to the end of the fourth paragraph in the Discussion: "All this having been said, it should be noted that the estimation of the directionality of neurofilament transport using our method is robust to bleaching errors (as it is for flanking window size) because it is given by the ratio of the slopes in the

flanking windows and any bleaching correction is a multiplier applied to both numerator and denominator in that calculation.”

19. In the legend of figure 1, the text claims that the image of healthy mitochondria is in the top image, whereas in the figure itself they are in the bottom image. I would suggest that in both panels D and E, the healthy axons should be on the top.

The figure legend has been corrected such that the unhealthy and healthy axons are referred to properly.

20. In line 621, the formula given is correct (but see comment above about tau) only for the central ROI. The formula for the flanking ROIs should be something like $F(t) = A(1 - \exp(-t/\tau))$.

The formula referred to here is for the pulse-escape experiment, which only uses a central window with an exponential decay formula. There are no flanking windows in this version of the experiment. Flanking windows are used in the pulse-spread experiment, but the increase in the fluorescence in the first few minutes in those windows is linear, not exponential (see above).

21. In line 643: the description of the quality of the objectives is relevant to the prior description of image flatness, rather than to image alignment. Move to the correct location.

The phrase regarding objective quality has been removed, as there is no other discussion of the impact of microscope components on image quality or flatness.

22. In. line 654: Couldn't the problem of the effect of improper alignment be somewhat alleviated by leaving buffer zones between the central and flanking ROIs?

Leaving buffer zones would not solve this problem because the flanking windows would not capture all the fluorescence that departed the activated region at short times, which is a necessary component of our analysis.

23. I did not find any discussion of how the methods help address the pausing nature of transport of neurofilaments. This point is raised by the authors as an advantage of the proposed techniques, but is not address in the methodology section or in the discussion.

The pulse-escape analysis, i.e. the decay of fluorescence in the center window, can yield predictions regarding the pausing behavior of the filaments. The analysis has been described in detail in a previous paper (LI et al. 2014). We have added citations to this paper to clarify this point.

Technical:

The quality of the images in the review document was quite poor, making it quite difficult to assess some of the details that the authors refer to. The text is pixelated and difficult to read. The morphology of the mitochondria in figure 2 is not readily discernible, especially in the case of the punctate ones. There are a few double periods (..) in the text.

There seems to have been an issue with the figures made available to the Reviewers, as our original files were 300dpi with a font size of at least 12. We have replaced the double periods.

To conclude - this technical description of the manner in which axonal transport of neurofilaments (or other structures) can be performed is interesting and described in good detail. The formal description of the analysis is not clear enough in my opinion.



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Supplemental Coding Files

`Alignment_by_fixed_region.py`

