Rapid movement of axonal neurofilaments interrupted by prolonged pauses

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Axonal cytoskeletal and cytosolic proteins are synthesized in the neuronal cell body and transported along axons by slow axonal transport, but attempts to observe this movement directly in living cells have yielded conflicting results. Here we report the direct observation of the axonal transport of neurofilament protein tagged with green fluorescent protein in cultured nerve cells. Live-cell imaging of naturally occurring gaps in the axonal neurofilament array reveals rapid, intermittent and highly asynchronous movement of fluorescent neurofilaments. The movement is bidirectional, but predominantly anterograde. Our data indicate that the slow rate of slow axonal transport may be the result of rapid movements interrupted by prolonged pauses.

Slow axonal transport refers to the anterograde movement of cytoskeletal and cytosolic proteins along axons at modal rates of 0.2–8 mm per day, a speed that is several orders of magnitude slower than the rate of transport of the membranous organelles that comprise fast axonal transport[1]. Most attempts to visualize slow axonal transport directly in living neurons have used fluorescence photobleaching or photoactivation to 'mark' a population of cytoskeletal proteins in axons, but these approaches have yielded conflicting results. The first study of this kind reported anterograde movement of tubulin in cultured PC12 cells[2], but subsequent studies on tubulin in PC12 cells[3], cultured chick sensory neurons[4] and developing neurons in grasshopper and zebrafish embryos[5,6], as well as studies of tubulin, actin and neurofilament protein in cultured mouse sensory neurons[7–10], have all failed to reveal movement.

Microtubules have been shown to move *en masse* in cultured embryonic frog neurons growing on laminin substrates[11,12], but Chang *et al.*[13] have shown that this movement is probably attributable to stretching of the growing axon rather than *bona fide* slow axonal transport because the movement is dependent on the nature of the substratum and is not observed when the neurons are induced to grow at comparable rates on more adhesive substrates. Direct observation of microtubules containing rhodamine-labelled tubulin has revealed movement within growth cones of cultured embryonic frog neurons[14] and in growth cones and developing axonal branches of cultured hamster cortical neurons[15], but neither of these studies revealed movement in the axon shaft because individual microtubules could not be resolved. Recently, Chang *et al.*[16] used fluorescence speckle microscopic analysis of Cy3-labelled tubulin to visualize microtubules within axons of cultured embryonic frog neurons, but they were still unable to detect any movement.

The failure of so many independent efforts to demonstrate the movement of cytoskeletal proteins in axons has generated considerable controversy concerning the mechanism of slow axonal transport[17–19]. Most of the controversy has centred on the form in which the cytoskeletal proteins move. According to the polymer-transport hypothesis, cytoskeletal polymers are the vehicle for slow axonal transport and cytosolic proteins move by association with the moving polymers. According to the subunit-transport hypothesis, cytoskeletal polymers are stationary, and cytoskeletal and cytosolic proteins move as free subunits or oligomeric complexes. The principal reason for the persistence of this controversy has been

the lack of an experimentally accessible system in which movement of cytoskeletal proteins can be observed directly in living cells.

Here we show that cultured neurons from the superior cervical ganglia of neonatal rats frequently exhibit natural discontinuities in the neurofilament array along their axons, resulting in short segments of axon that lack neurofilaments. These gaps have offered us a unique opportunity to visualize the movement of a cytoskeletal protein in living cells without the need for special approaches such as photobleaching or photoactivation. If axonal neurofilaments all move at the same rate (that is, in a synchronous manner), then we would expect the entire gap to translocate along the axon. Conversely, if neurofilaments move at a broad range of rates (that is, in an asynchronous manner), then we would not expect the gaps to translocate, but we would expect to observe individual neurofilaments moving through them.

To test these hypotheses, we transfected cultured rat sympathetic neurons by nuclear injection of a plasmid coding for green fluorescent protein (GFP) linked to the amino terminus of neurofilament protein M (NFM). We show that the GFP domain does not interfere with the assembly of the NFM and that the GFP–NFM fusion protein incorporates into neurofilament polymers throughout the axonal arbor. Live-cell imaging of the GFP fluorescence reveals that fluorescent filaments move rapidly through the gaps at
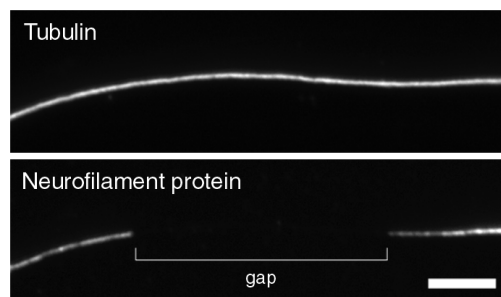

Figure 1 Cultured sympathetic neurons exhibit naturally occurring gaps in the axonal neurofilament array. Immunofluorescence of an axon double-stained with antibodies specific for β-tubulin (present in all microtubules) and neurofilament protein L (NFL; present in all neurofilaments). Note the gap in the NFL staining; this gap represents a region of axon that lacks neurofilaments. Gaps in the microtubule array are never encountered. Scale bar represents 5 μm.
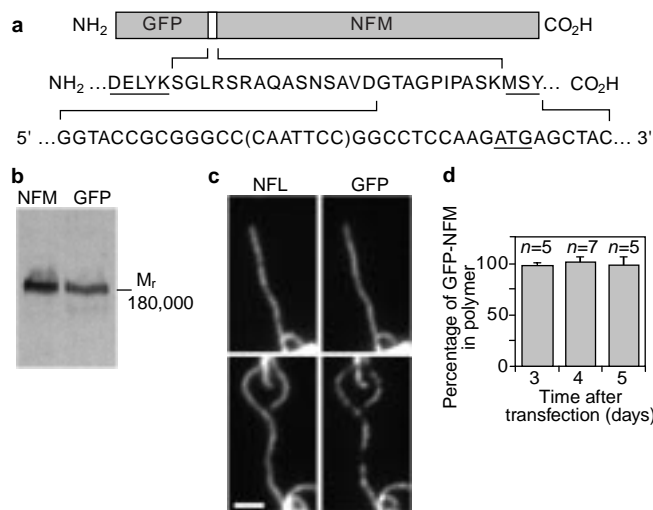
Figure 2 Characterization of the GFP–NFM fusion protein. a, Diagram of GFP–NFM, showing the amino-acid (NH_2 to CO_2H) and nucleotide (5′ to 3′) sequences at the fusion junction. The underlined amino-acid sequences represent the C-terminal end of GFP (left) and the N-terminal end of NFM (right). The nucleotide sequence in parentheses was inserted during the cloning process. The sequence to the left of the parentheses is part of the multiple cloning site of the parent plasmid, pEGFP–C1, and the sequence to the right of the parentheses is the 5′ end of the rat NFM cDNA (start codon of NFM is underlined). **b**, Transfection of COS-7 cells with pEGFP–NFM results in expression of the full-length GFP–NFM fusion protein. Western blot showing single protein staining with antibodies specific for NFM and GFP. The apparent relative molecular mass (M_r) of the protein is consistent with a fusion of rat NFM (apparent M_r ~145,000 by SDS–PAGE) to GFP (M_r ~27,000) separated by a linker region (M_r ~3,000). **c**, Visualization of GFP–NFM in single neurofilaments by fluorescence microscopy. Axonal neurofilaments were induced to splay apart from each other by extraction of neurons with 0.5% Triton X-100 in the presence 0.2 M NaCl 3 days after transfection[20]. NFL was detected by immunofluorescence and GFP–NFM was detected directly without immunostaining. GFP–NFM incorporation was continuous along some filaments (top) and discontinuous along others (bottom). Scale bar represents 1.5 μm. **d**, Quantification of the proportion of GFP–NFM in polymer. Fluorescence intensity was quantified in axons before and after permeabilization with 0.02% saponin at 3, 4 and 5 days after transfection. Error bars represent s.d.

peak rates of up to 2.3 μm s^{-1} in an intermittent and highly asynchronous manner, and in a predominantly anterograde direction. These data provide direct support for the polymer-transport hypothesis of slow axonal transport, and they indicate that the slow average rate of slow axonal transport can be accounted for by rapid movements interrupted by prolonged pauses.

Results

Naturally occurring gaps in the axonal neurofilament array. Cultured neurons from the superior cervical ganglia of neonatal rats frequently exhibit gaps in the neurofilament array along their axons (Fig. 1). These gaps appear to occur naturally in these cultures and are most common in the thinnest axons, presumably because they contain the fewest neurofilaments. We have never encountered gaps in the neurofilament array in cultured neurons from dorsal root ganglia, which have a much higher neurofilament content[20]. The gaps are unlikely to have any functional significance for cultured neurons, because neurons can survive and function even in the complete absence of neurofilaments[21,22]. Nevertheless, the gaps do represent a special opportunity to investigate the axonal transport of neurofilament protein.

Expression of the GFP–NFM fusion protein. To visualize neurofil-
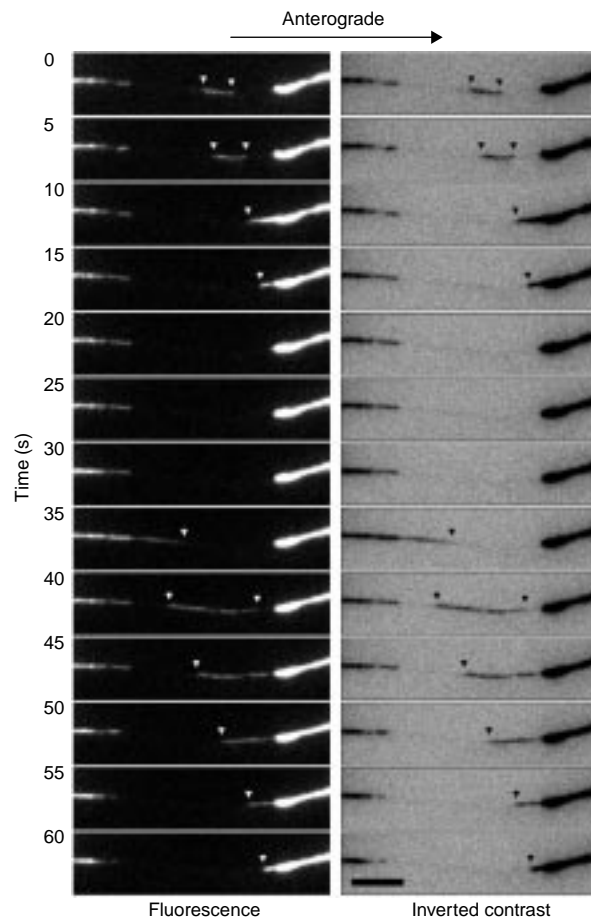

Figure 3 Movement of neurofilaments through a gap in the axonal neurofilament array. Time-lapse images of the GFP fluorescence were acquired at 5-s intervals. The images of the GFP fluorescence (on the left) are also displayed with inverted contrast (on the right) for greater clarity. The sequence begins with a short filament in the gap; this filament moves out of the gap in an anterograde direction (average velocity = 0.32 μm s^{-1}; peak velocity = 0.64 μm s^{-1}). Subsequently, a longer filament moves through the same gap, also in an anterograde direction (average velocity = 0.52 μm s^{-1}; peak velocity = 1.26 μm s^{-1}). Arrowheads mark the leading and trailing ends of the filaments, where visible. Scale bar represents 5 μm. See Supplementary Information for a movie of this time-lapse sequence plus extra footage.

aments in living neurons, we constructed a plasmid (pEGFP–NFM) which encodes GFP linked to the N terminus of NFM (Fig. 2a). Transfection of COS-7 cells with pEGFP–NFM resulted in the expression of a protein with an apparent relative molecular mass (as judged by SDS–polyacrylamide gel electrophoresis (SDS–PAGE)) of 180,000; this protein was recognized by antibodies specific for either NFM or GFP (Fig. 2b). Thus pEGFP–NFM directs the expression of the full-length GFP–NFM fusion protein.

Visualization of neurofilaments in cultured neurons. We transfected primary cultures of dissociated rat sympathetic neurons with pEGFP–NFM by nuclear injection. Green fluorescence appeared in the cell body and proximal axons within 1 day after transfection, and by 3 days it had extended into the most distal axons. Immunofluorescence microscopy of splayed axonal cytoskeletons showed that the GFP–NFM was present in neurofilaments throughout the axonal arbor (Fig. 2c), and ≥96% of the GFP–NFM remained in the axons after permeabilization with saponin (Fig. 2d), a result consistent with biochemical studies that have shown that >95% of neurofilament proteins are polymerized in these neurons[23].

Transfection experiments in SW13cl.2vim⁻ cells, which lack cytoplasmic intermediate filaments, showed that GFP–NFM alone did not assemble into filaments, but it did assemble in the presence of neurofilament protein L (data not shown). This result is consistent with previous studies that have shown that neurofilaments are obligate heteropolymers *in vivo*[24,25]. Fusion of GFP to the carboxy terminus of NFM resulted in the expression of a fusion protein that formed non-filamentous aggregates (data not shown); we believe that this may explain the punctate non-filamentous distribution of C-terminally fused GFP–NFM observed by Yabe *et al.*[26]. These data indicate that the presence of the GFP domain at the N terminus of NFM does not interfere with its assembly properties, and that the N-terminally fused fusion protein assembled into neurofilaments throughout the neuron.

Live-cell imaging of gaps in the axonal neurofilament array. To investigate the movement of neurofilaments, we located gaps in the GFP–NFM fluorescence along axons of neurons 3 days after transfection. Time-lapse imaging revealed the movement of fluorescent filaments through the gaps (Fig. 3; see Supplementary Information). We never observed movement of the entire gap, although the movements of the filaments sometimes caused the gaps to lengthen, shorten or disappear while we were observing them (see Supplementary Information). Filament movements were not confined to gaps in the neurofilament array: we were frequently able to detect the movement of filaments in neurofilament-containing flanking regions as filaments moved into or out of the gaps (for example, see Supplementary Information). In addition, we were sometimes able to detect the movement of filaments in axons that did not contain gaps if the axons happened to contain very few neurofilaments (data not shown).

To characterize the movement of the filaments, we acquired time-lapse movies of 47 gaps in 21 cells. The gaps ranged from 2 to 54 μm in length and the duration of observation ranged from 4.9 to 10.4 min. 77% of the gaps showed movement during the observation period. On average, we observed one moving filament per 5.1 min of observation. Inspection of axons by differential interference contrast microscopy during and after time-lapse imaging revealed healthy-looking axons and growth cones and rapid bidirectional movement of membranous organelles, indicating that there was no gross perturbation of the axon associated with excitation of the GFP during epifluorescence illumination.

Analysis of filament movement. We analysed the movement of all fluorescent structures that exhibited a net displacement of at least 10 pixels (0.685 μm) during the observation period and that could be tracked unambiguously for at least three consecutive frames. The fluorescent filaments represented 95% (n=69) of the moving structures and ranged from 1.0 to 15.8 μm in length (mean = 4.1 μm, n= 64; Fig. 4c). The width of the filaments was 0.3±0.06 μm (mean±s.d., n= 17), which is comparable to the diffraction-limited width of single neurofilaments in splayed axonal cytoskeletons (0.3 ± 0.03 μm, mean±s.d., n=17; Fig. 2c)[20]. The remaining 5% (n=4) of the moving structures were punctate in appearance and had a diameter of 0.4±0.09 μm (mean±s.d., n=4). Three of these structures were observed in the same gap and moved anterogradely; the other one was observed in a different gap and moved retrogradely. We sometimes observed punctate GFP–NFM incorporation along single neurofilaments in splayed axonal cytoskeletons, so it is possible that these fluorescent structures represented discrete sites of GFP–NFM incorporation within neurofilaments. The motile behaviour of the punctate structures was similar to that observed for the filaments, but we did not observe a sufficient number of them to allow statistical analysis.

The movement of the fluorescent filaments was bidirectional, but 83% moved in a net anterograde direction. Movements in both directions were rapid, but were frequently interrupted by prolonged pauses (Fig. 4a). For the 69 filaments whose movements we tracked, the average time spent pausing was 73%. The average velocity excluding these pauses ranged from 0.02 to 1.21
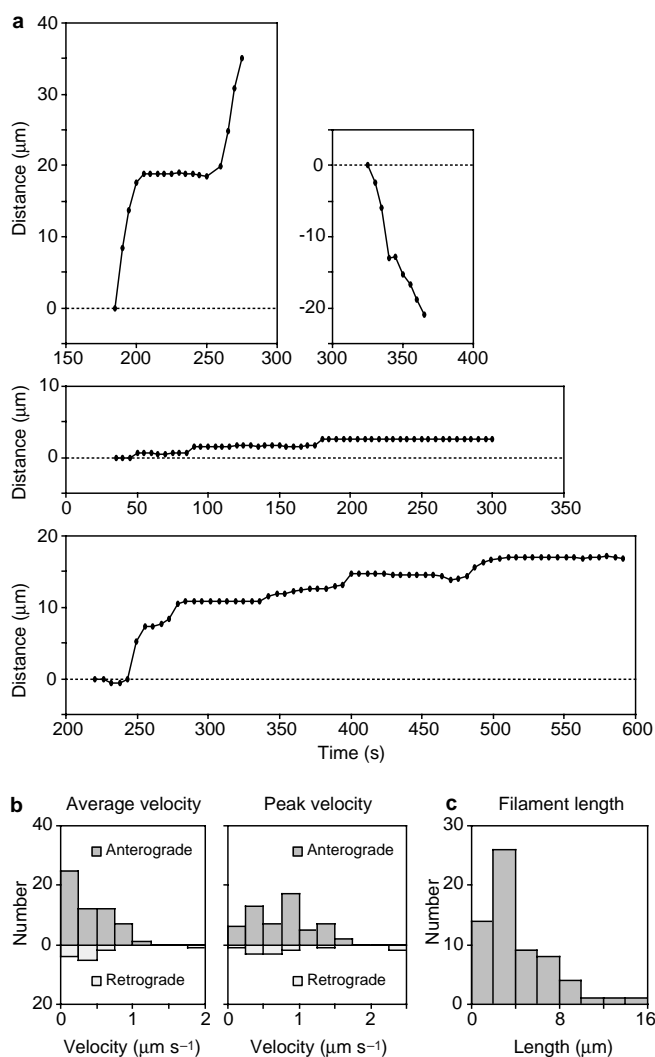

Figure 4 **Analysis of moving filaments. a**, Movements of four representative filaments: an anterogradely moving filament interrupted by a single lengthy pause (top left); a retrogradely moving filament interrupted by a single brief pause (top right); and two anterogradely moving filaments interrupted by multiple pauses of varying duration (bottom). Each point represents the distance of the filament from the starting position, measured along the axon. Anterograde and retrograde movements are represented as positive and negative displacements, respectively. The abscissa represents the time elapsed since the start of the movie. **b**, Histograms of average velocities (excluding pauses) and peak velocities for all 69 filaments. A filament was considered to have paused if it moved at less than one pixel per s (0.0685 μm s⁻¹), which we estimate to be the precision limit of our measurements. **c**, Histogram of filament lengths. Five of the 69 filaments were not measured because both ends were never visible in the same image.

μm s⁻¹ in the anterograde direction (mean = 0.38 μm s⁻¹ , n = 57) and from 0.15 to 1.82 μm s⁻¹ in the retrograde direction (mean = 0.49 μm s⁻¹, n = 12; Fig. 4b). The net average velocity for all filaments excluding the pauses (and considering retrograde velocities as negative and anterograde velocities as positive) was 0.23 μm s⁻¹ in the anterograde direction. The peak velocity ranged from 0.17 to 1.69 μm s⁻¹ in the anterograde direction (mean = 0.74 μm s⁻¹, n = 57) and from 0.21 to 2.28 μm s⁻¹ in the retrograde direction (mean = 0.89 μm s⁻¹, n = 12; Fig. 4b). The rapid and intermittent movement of these neurofilaments is not consistent with models of slow axonal transport that assume a slow and synchronous move-

ment of a network of crosslinked cytoskeletal polymers[27]. Instead, these data indicate that the movement of the cytoskeletal polymers is highly asynchronous, with individual neurofilaments moving independently of each other at a broad range of rates[28].

Discussion

The molecular mechanism of slow axonal transport has eluded researchers for more than two decades and has been the subject of considerable controversy[17–19]. Our observations of naturally occurring gaps in the axonal neurofilament array show that neurofilament polymers move in axons and this provides direct support for the polymer-transport hypothesis of slow axonal transport. A more general test of this hypothesis will also require direct observation of the movement of tubulin and actin in axons, but this will require different experimental approaches because gaps are not encountered in the microtubule and microfilament arrays of axons.

The retrograde movements of neurofilaments that we observed are unexpected because it has generally been assumed that slow axonal transport is exclusively anterograde. It seems unlikely that these retrograde movements represent elastic recoil of anterogradely moving filaments, because the movements were often sustained and were frequently interrupted by pauses. Both anterogradely and retrogradely moving filaments often exhibited brief reversals, but we were unable to track them for sufficient periods of time to determine whether the retrogradely moving filaments eventually moved in a net anterograde direction, or whether they continued to move in a net retrograde direction. Thus the functional significance of the retrogradely moving filaments for slow axonal transport remains to be determined.

Studies of slow axonal transport using radioisotopic pulse labelling have shown that neurofilament proteins move anterogradely in axons at an average rate of 0.25–3 mm per day ($0.003–0.035 \mu m s^{-1}$) depending on the organism and the neuronal cell type[28,29]. Our live-cell observations indicate that the actual rate of neurofilament movement is considerably faster than these average rates, but that the movements are interrupted by prolonged pauses. The proportion of the time that the filaments we analysed spent pausing was 73%, but this is certainly an underestimate for the entire neurofilament population because we confined our analyses to filaments that moved and excluded filaments that paused throughout the period of observation. However, if we assume an average transport rate of 0.25–3 mm per day for the entire neurofilament population (from radioisotopic pulse-labelling studies) and an average transport rate of $0.23 \mu m s^{-1}$ for the individual neurofilaments (from our live-cell observations), then we calculate that neurofilaments must actually spend an average of 85–99% of their time pausing during their journey along the axon.

These data indicate that the slow rate of slow axonal transport may be the result of rapid but relatively infrequent movements. In contrast to fast axonal transport, which is a highly efficient process characterized by rapid and continuous movements, slow axonal transport appears to be a relatively inefficient process in which the filaments also move rapidly, but spend only a small proportion of their time moving. This new motile behaviour could explain why it has proven to be so difficult to observe the movement of neurofilaments and other cytoskeletal polymers in nerve cells. □

Methods
Cell culture.
Neurons dissociated from superior cervical ganglia of neonatal (P0–P1) rats were plated onto glass coverslips coated with poly-lysine and Matrigel (Collaborative Research, 10 μg ml⁻¹). Cultures were maintained at 37 °C in Liebovitz's L-15 medium (Gibco) supplemented with 0.6% glucose, 2 mM L-glutamine, 100 ng ml⁻¹ 2.5S nerve growth factor (Collaborative Research), 10% adult rat serum and 0.5% hydroxypropylmethylcellulose (Methocel, Dow Corning)[30]. COS-7 cells were cultured at 37 °C with 5% CO₂ in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum.

Cloning and transfection.
A blunt-ended *Eco*RI fragment of pRSVi-NFM containing the full-length complementary DNA encoding

rat NFM (Genbank accession number M18628)[31] was subcloned into the *Apa*I site of the pEGFP–C1 plasmid vector (Clontech; Genbank accession number U55763) and purified by CsCl centrifugation. COS-7 cells were transfected using GenePORTER transfection reagents (Gene Therapy Systems). Neurons were transfected 2 days after plating by pressure injection of purified plasmid (300 μg ml⁻¹ in 50 mM potassium glutamate, pH 7.0) directly into the nucleus[32]. In most experiments the plasmid was co-injected with 1.25 mg ml⁻¹ tetramethylrhodamine dextran (of relative molecular mass 10,000; Sigma) to allow visual confirmation of the injection procedure. 10–20% of the cells survived and expressed green fluorescence 3 days after injection.

Cell extraction and immunoblotting.
The transfected cells were lysed in 10 mM Tris-HCl (pH 7.5) and 1% SDS, boiled in SDS–sample buffer, centrifuged to remove insoluble materials, and subjected to SDS–PAGE. Western blots were immunostained with mouse monoclonal antibodies specific for NFM (Sigma, clone NN18) and GFP (Clontech), using enhanced chemiluminescence detection reagents (Amersham).

Live-cell imaging and image analysis.
Cells were observed 3–5 days after transfection in a sealed chamber containing oxygen-depleted culture medium[15]. The medium was depleted of oxygen by preincubation in an air-tight syringe for 2 h at 37 °C in the presence of a 1:100 dilution of EC-Oxyrase (Oxyrase), 20 mM sodium succinate and 20 mM sodium DL-lactate[33]. Before assembling the chamber, we filtered the medium through a 0.2-μm syringe filter to remove EC-Oxyrase particulates. Cells maintained in the sealed chambers retained active motile growth cones and apparently normal organelle movements for many hours, consistent with reports that the energy metabolism of cultured neurons is predominantly glycolytic[34]. Cells were observed by epifluorescence and differential interference contrast microscopy using a Nikon Diaphot 300 microscope, and maintained at 37 °C on the microscope stage using a Nicholson Air Stream Incubator. For time-lapse imaging, the epifluorescence illumination was attenuated to 10% or 15% using neutral density filters, and images were acquired with 1-s exposures at 5-s intervals using a Nikon ×100/1.4 NA Plan Apo oil immersion objective, a fluorescein isothiocyanate (FITC)/enhanced GFP (EGFP) filter set (Chroma Technology, HQ 41001), and a Photometrics cooled charge-coupled-device (CCD) camera with a Kodak KAF 1400 chip. Motion was analysed by tracking the position of the leading and trailing ends of the filaments in successive time-lapse image frames. Image processing and analysis procedures were done on a Macintosh computer using Oncor-Image software (Oncor) and on a PC using MetaMorph software (Universal Imaging).

Immunofluorescence microscopy and detergent extraction.
Cultures were fixed with formaldehyde and stained using a polyclonal antibody specific for NFL and a monoclonal antibody specific for β-tubulin, as described[20]. To determine the proportion of GFP–NFM in polymer form, we permeabilized cells with 0.02% saponin in a solution composed of 60 mM sodium PIPES, 25 mM sodium HEPES, 0.19 M NaCl, 10 mM sodium EGTA, 2 mM MgCl₂, pH 6.9 (ref. 30), and quantified the fluorescence intensity in the axons before and after permeabilization using the segmented mask method[35].

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