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Live-cell imaging of slow axonal transport in cultured neurons

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Abstract

Cytoskeletal polymers and other cytosolic protein complexes are transported along axons in the slow components of axonal transport. Recent studies on the movement of neurofilaments and microtubules in the axons of cultured neurons indicate that these polymers actually move at fast rates and that the movements are also infrequent and highly asynchronous. These observations indicate that the slow rate of slow axonal transport is due to rapid movements interrupted by prolonged pauses, which presents special challenges for studies on the mechanism of movement. This chapter describes the procedures that the author's laboratory has used to observe and analyze the movement of neurofilaments and microtubules in axons of cultured neurons from the superior cervical ganglia of neonatal rats. In particular, the author describes how to culture these neurons, how to transfect them by nuclear injection, and how to detect the rapid and infrequent movement of cytoskeletal polymers using time-lapse fluorescence imaging.

I. Introduction

Cytoskeletal and cytosolic proteins are synthesized in the neuronal cell body and transported along axons in the slow components of axonal transport at average rates of approximately 0.3-3 mm/day (Lasek *et al.*, 1984). Recently we and others have succeeded in observing the slow axonal transport of neurofilaments and microtubules directly in cultured nerve cells using live-cell fluorescence imaging (Wang *et al.*, 2000; Roy *et al.*, 2000; Wang and Brown, 2001; Wang and Brown, 2002). Contrary to expectations, these cytoskeletal polymers move at rapid rates, approaching the rate of fast axonal transport, but their movements are also infrequent and highly asynchronous. Based on these observations, we have proposed that slow axonal transport is powered by fast motors and that the overall slow rate is due to rapid movements interrupted by prolonged pauses (Brown, 2000).

In spite of this recent progress, there is still a great deal to be learned about the mechanism of slow axonal transport. For example, what are the structural forms in which cytoskeletal and cytosolic proteins move, what are the motors that move them, what are the tracks along which they move, and how is their movement regulated? Cultured neurons offer researchers a special opportunity to address these questions because of their amenability to observation by live-cell fluorescence imaging. In this article, I describe two approaches that have allowed us to observe the axonal transport of cytoskeletal proteins in cultured neurons. One approach takes advantage of naturally occurring gaps in the axonal neurofilament array in certain types of cultured neuron, and the other employs fluorescence photobleaching. While the former approach is only applicable to studies on neurofilaments, the latter approach has also permitted us to observe the axonal transport of microtubules, and we believe that this

technique should also be capable of detecting the movement of other cytoskeletal and cytosolic proteins in axons.

II. Culturing neurons from superior cervical ganglia

A. Establishing cultures

All of our live-cell imaging studies on slow axonal transport have been performed using primary cultures of dissociated neurons from superior cervical ganglia (SCG). A more detailed discussion of the culture of these neurons can be found elsewhere (Hawrot and Patterson, 1979; Johnson *et al.*, 1981; Johnson and Argiro, 1983; Higgins *et al.*, 1991; Mahanthappa and Patterson, 1998). We have most experience with SCG neurons from rats, but the procedures described here work equally well for mice. We purchase timed pregnant female Sprague Dawley rats from Harlan on a weekly basis and sacrifice the newborn rat pups using carbon dioxide followed by cardiac puncture. We normally use neonatal pups, but older pups can also be used. The two ganglia obtained from a single pup are sufficient for one batch of cultures because we plate the cells at very low density in order to facilitate tracing of individual axons along their length.

All procedures are performed under sterile conditions in a horizontal laminar flow hood. Hoods with a vertical flow design are not suitable because they cannot accommodate a stereo dissection microscope. The ganglia are dissected from the animal together with the associated carotid artery and vagus nerve as described by Higgins *et al.* (1991) and then immersed in a 35 mm plastic dish containing Leibovitz's L-15 medium. The associated tissue is removed under the dissection microscope using fine forceps and the cleaned ganglia are transferred to a 15 ml disposable polystyrene centrifuge tube containing fresh L-15 and allowed to settle to the bottom of the tube under gravity. If any of the submerged ganglia adhere to the sides of the tube, they can be dislodged by squirting L-15 on them using a Pasteur pipette. Contamination of the cultures with non-neuronal cells can be reduced by removing the thin connective tissue capsule that ensheaths the ganglia, but we do not find this to be necessary when using L-15 based culture media (see below).

To dissociate the neurons, we rinse the ganglia once with phosphate-buffered saline (PBS) and then incubate them in 2.5 mg/ml collagenase (Type 3, Worthington Biochemicals) at 37°C for 1 hour. We then rinse them twice with PBS and incubate them in 2.5 mg/ml trypsin (3x crystallized, Worthington Biochemicals) at 37°C for 30 minutes. The enzyme solutions are prepared fresh in PBS and sterilized by 0.2 µm syringe filtration prior to use. For the rinses, the ganglia are allowed to settle to the bottom of the tube under gravity and then the liquid is removed using a Pasteur pipette, leaving just enough to cover the ganglia so that they do not dry out. After trypsinization, the ganglia are rinsed with L-15 containing 15-20% fetal bovine serum (to inactivate the trypsin), and then triturated in 2 ml L-15 containing 0.5 mg/ml bovine serum albumin ("L-15 + BSA") using a Pasteur pipette (5-10 passages). The resulting cell suspension is diluted in L-15 + BSA to the desired concentration and plated onto coverslips in the culture dish assemblies described below (150 µl cell suspension per well). It is important not to triturate too much because this can reduce the yield of viable cells. The actual cell density can be determined using a hemocytometer, but we find it more convenient to estimate it in units of ganglia/ml. In our hands, a concentration of dissociated cells equivalent to 0.03 to 0.06 ganglia/ml yields approximately 25-50 neurons per dish. At this density, two ganglia yield enough cells to plate more than 100 dishes, though we typically plate only 8-12. The cells are allowed to settle onto the coverslip under gravity for 45 minutes in the incubator (37°C, atmospheric CO₂) and then 1.5 ml culture medium (see below) is added to each dish. The cells can be maintained for at least a week without changing the medium, providing that care is taken to minimize evaporation loss, but we generally replace the medium with fresh medium every 3-4 days. Prior to adding fresh

medium, we aspirate the old medium from the dish but not the well (about 85 μ l), so as to avoid disturbing the cells on the coverslip. More frequent feeding is not necessarily better, since some conditioning of the medium may occur with time in culture.

B. Culture medium

The culture medium that we use is based on the formulation of Bray (1991) and contains 10% adult rat serum, 0.6% glucose, 2 mM L-glutamine, 0.5% hydroxypropylmethylcellulose (Methocel™ F4M-PREM, Dow Chemical Co.) and 50 ng/ml nerve growth factor (2.5S subunit purified from mouse salivary glands; Collaborative Research) in L-15 (phenol red free, Gibco). L-15 medium is designed to buffer its pH in atmospheric CO₂ so it is more convenient for observation and microinjection of cells on the microscope stage than media with bicarbonate buffering systems. An additional benefit is that non-neuronal cells proliferate far less in this medium than in bicarbonate buffered media, thereby eliminating the need for the addition of anti-mitotic agents to the culture medium. We avoid using phenol red in the culture medium because it increases the background fluorescence during live-cell imaging. The fluorescence of the culture medium may be reduced further by using a custom L-15 formulation lacking riboflavin, but we have not tried this ourselves. With good sterile technique it is not necessary to use antibiotics. A defined serum-free L-15 based culture medium can also be used (Hawrot and Patterson, 1979; Mahanthappa and Patterson, 1998), but the neurons appear to do better in the presence of serum when cultured at low density. While it is possible to culture SCG neurons for many months, all of our own work has been performed within one week of plating.

Adult rat serum can be prepared by the method of Hawrot & Patterson (1979) or purchased from a commercial source such as Harlan. We sterilize the serum by 0.2 μ m syringe filtration and store it frozen in aliquots to minimize repeated freezing and thawing. Often the serum forms a fine precipitate after a few days at 37°C. This precipitate can be mistakenly identified as microbial contamination, but it is actually innocuous and does not harm the cells. With serum from some commercial sources, the precipitate can become so dense that it obscures the cells. In this case, switch to a different commercial source or prepare the serum yourself. Fetal bovine serum can also be used, but it is our impression that the cells are healthier and attach better to the substrate in the presence of adult rat serum.

The function of the Methocel™ is to increase the viscosity of the culture medium. It is not critical, but the cells appear to attach better to the substrate when it is present. Since Methocel™ solutions are too viscous to sterilize by filtration, the powder must be autoclaved. We weigh out 200 mg aliquots of Methocel™ powder in 50 ml disposable polypropylene centrifuge tubes and then sterilize by autoclaving with the caps loosely attached. After cooling, the caps can be tightened and the dishes can be stored indefinitely at room temperature. To dissolve the Methocel™, we add 40 ml sterile L-15 and shake the tube overnight at room temperature. The resulting solution contains insoluble particulate material, which we remove by filtration using a 5 μ m syringe filter (Millex-SV, Millipore).

C. Culture dishes

The neurons are cultured on glass coverslips in the coverslip culture dish assembly described by Bray (1991) (Fig. 1). To prepare the dishes, we drill 13/32" diameter holes in 35 mm polystyrene culture dishes using a titanium-coated step drill (McMaster-Carr Supply Co.). The drill bit should be cleaned thoroughly prior to use since it contacts the culture dish. We use Nunclon 35 mm culture dishes with vented lids (Nalge Nunc), but any similar dish should be suitable. It is important to use slow and steady pressure with a sharp drill bit and to support the dish from underneath to avoid cracking the plastic. We use a specially built jig milled out of aluminum to hold the dish in position on the drill press. The drill often leaves a

slight burr, which must be removed by gently scraping around the holes with a scalpel blade in order to allow coverslips to be placed flush against the plastic (see below).

The coverslips are acid-washed prior to use by soaking them in concentrated nitric acid for 18 hours and then rinsed extensively with water. We use 22 × 22 mm No. 1.5 coverslips, which match the 0.17 mm coverslip thickness correction of our objectives. To dry the coverslips, we dip them in ethanol and then flame them. To attach the coverslips to the dishes, we invert the dishes so that the base faces upwards and apply a circle of molten paraffin wax around the hole using a paint brush. After the paraffin has cooled, we place a coverslip over the hardened paraffin and hold the inverted dish with the coverslip on it close to the surface of an inverted hot plate. As the paraffin beneath the coverslip melts, it spreads out between the coverslip and the dish. Once the paraffin has melted fully, the dish is removed from the heat and cooled immediately using a gentle stream of compressed air. Be careful not to apply the paraffin too close to the hole or to use too much paraffin or too much heat because this may cause the paraffin to flow into the well. Paraffin works well because it is not toxic to the cells and it remains hard at 37°C, yet the coverslip can be removed if necessary using a thin razor blade. Silicone adhesives such as aquarium sealant can also be used (Challacombe *et al.*, 1997), but we have not tried this ourselves. Coverslip culture dishes are now also available commercially (World Precision Instruments & Warner Instruments).

On the day before the dissection, the coverslip culture dishes and lids are sterilized with 70% ethanol for 45 minutes. After air-drying in the hood, the coverslips are treated with 1mg/ml poly-D-lysine HBr (M_w 70-150,000, Sigma) in sodium borate buffer, pH 8.5, for 3 hours (150 μ l per well) as described by Higgins *et al.* (1991). We make up a 10 mg/ml stock solution in borate buffer and freeze it in aliquots. Prior to use, the aliquots are diluted in borate buffer and sterilized by 0.2 μ m syringe filtration. After the poly-lysine treatment, the coverslips are rinsed three times with sterile water to remove poly-lysine that is not bound to the glass. A few milliliters of sterile water is then added to each dish and they are stored overnight. On the morning of the dissection, the coverslips are rinsed three more times with sterile water, and then coated with 10 μ g/ml mouse laminin (BD Biosciences) in L-15 or 10 μ g/ml phenol red-free Matrigel™ (BD Biosciences) in L-15. Matrigel is a basement membrane extract that is produced by Engelbreth-Holm-Swarm mouse tumor cells. It is an excellent substrate for SCG neurons, but we prefer not to use it for immunofluorescence applications because it does tend to form sticky clumps. The laminin and Matrigel™ are stored in aliquots at –80°C. To prevent gelation, they are thawed on a slurry of ice and water on the day of the dissection, and diluted into ice-cold L-15. After dilution, the L-15 is allowed to warm to room temperature. We apply 150 μ l of laminin or Matrigel™ solution per coverslip well and incubate for 4 hours in the incubator (37°C, atmospheric CO₂). The dissection is performed during this incubation period and then the coverslips are rinsed once with L-15 before plating.

III. Transfecting neurons by nuclear injection

Primary neurons are not readily transfected using many of the protocols that work well for mitotic cells. For example, when we have attempted to transfect cultured SCG neurons with cationic lipid reagents (e.g. Lipofectamine 2000, Gibco), we have obtained far fewer than one fluorescent cell per dish (unpublished observations). The principal reason for this appears to be that plasmids are not readily taken up by interphase nuclei. Better transfection efficiencies may be obtained with other approaches such as infection with viral or retroviral vectors or transfection using electroporation or biolistics (discussed elsewhere in this volume). Which method is most suitable for a particular application will depend on the number of transfected cells required for the study, the relative ease or difficulty of the

procedure, and the extent to which it is tolerated by the particular neuronal cell type being used. For studies on slow axonal transport in cultured neurons, other researchers have had success using infection with adenoviral vectors (Roy *et al.*, 2000) and transfection with calcium phosphate (Ackerley *et al.*, 2000). In my laboratory, we have had good success with microinjection of expression constructs directly into the cell nucleus (Garcia *et al.*, 1992; Wang *et al.*, 2000; Wang and Brown, 2001). This procedure has the advantage that it is simple, direct and reliable. By minimizing the number and relative proximity of the injected cells in each dish, we can ensure that we are able to trace single axons of expressing cells for long distances without ambiguity. The principal disadvantages of this approach are that it requires an inverted microscope equipped with a pressure injection apparatus and a micromanipulator, and that it requires a certain amount of practice to perfect the technique. Nuclear injection is also not suitable for transfection of large numbers of cells, but this is not a limitation for our live-cell imaging studies because a considerable amount of data can be obtained from a small number of cells.

For studies on the axonal transport of neurofilaments, we use a plasmid (pEGFP-NFM) that directs the expression of a green fluorescent fusion protein. This plasmid was produced by cloning the full length cDNA for rat neurofilament protein M (NFM) into the multiple cloning site of the Clontech pEGFP-C1 mammalian expression vector (Wang *et al.*, 2000). The resulting fusion protein (GFP-NFM) consists of green fluorescent protein (GFP) linked to the amino terminus of NFM by a short linker. For studies on microtubules, we have also recently experimented with a GFP-tubulin fusion protein construct that is commercially available from Clontech (pEGFP-Tub). Both vectors use the human cytomegalovirus (CMV) immediate early promoter and the SV40 early mRNA polyadenylation signal, and both express in cultured SCG neurons transfected by nuclear injection. We have no experience with other promoters or expression vectors at this time. The plasmids are purified using a Qiagen Endo-free Maxi Prep kit and the purified DNA is stored at 1-2 mg/ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) in aliquots at -20°C. Prior to injection, the DNA is diluted to 0.6 mg/ml in TE or 50 mM potassium glutamate, pH 7.2. The cells appear to tolerate injection of either buffer with no apparent adverse effects. During the plasmid preparation we take care to use disposable plastic whenever possible. If it is necessary to re-use plastic ware such as centrifuge tubes, care should be taken to remove all traces of detergent after washing. We wash our centrifuge tubes with ES-7X detergent (ICN Biomedicals) and rinse thoroughly.

We currently inject using an Eppendorf Injectman NI2 semi-automated injection system, but for many years we used a simpler and less expensive manual system comprised of a Narashige 3-axis hydraulic manipulator and Medical Systems Corporation PLI-100 pressure injector (Harvard Apparatus). The Eppendorf system uses internally generated compressed air as the pressure source and the Medical Systems Corporation system uses an external source of compressed nitrogen gas. Both systems work well. The Eppendorf system is more complex to operate but, once mastered, it can result in more reliable penetration of the plasma and nuclear membranes because it can be programmed to advance into the cell at rates of up to 7.5 mm/sec. We perform our injections using a Nikon TE300 inverted microscope situated on a vibration isolation table (Technical Manufacturing Corporation). We pull our own micropipettes from thick-wall borosilicate glass (1.0 mm OD, 0.58 mm ID, 4" long with internal filament; World Precision Instruments) using a Sutter P-97 Flaming-Brown Micropipette Puller (Sutter Instrument Co.). The internal glass filament facilitates back-loading of the micropipettes by capillary action. The puller settings must be determined empirically for a given filament geometry, and they may vary with ambient temperature and humidity. The goal is to obtain a tip that is fine enough to penetrate nuclei but not so fine that it frequently becomes blocked. We pull micropipettes with a two-stage pull cycle in order to obtain a short taper. The glass can be sterilized with dry heat (170°C

for 3 hours) prior to pulling, but we do not find this to be necessary. A container to hold glass capillary tubing during dry heat sterilization is commercially available (World Precision Instruments). To load the micropipette, we place approximately 1 μ l of injection solution at the opening of the capillary (the end opposite to the micropipette tip) and then suspend the micropipette vertically in a moist environment for a few minutes to allow the solution to draw down the capillary to the tip.

The micromanipulator is orientated so that the micropipette can be advanced axially into the cell at an angle of about 45 degrees to the horizontal. Since we mount the micromanipulator on the left hand side of the microscope, it is easiest for us to inject nuclei that are located on the left side of the cell body. If necessary, the culture dish can be rotated on the microscope stage to re-orientate the cell body prior to injection. We typically inject cells 18-48 hours after plating and observe them 1-3 days later. We inject under phase contrast optics using a 40x objective and a 0.52 NA long working distance condenser, which provides sufficient clearance for the micropipette. It is necessary to remove the lid from the culture dish for injection, but this rarely results in microbial contamination. If the cells are to be kept out of the incubator for more than 20 minutes, we warm the dish and microscope stage to 37°C using an air stream incubator (see Section V. C.). To prevent evaporation loss, we pipette a layer of low viscosity silicone fluid (0.75 ml dimethylpolysiloxane, 5 centistokes, Sigma) over the culture medium. The silicone fluid is pre-warmed to 37°C and sterilized using a 0.2 μ m nylon syringe filter. After injection, we generally remove the silicone fluid and the culture medium by aspiration and replace with fresh medium. This may reduce the chance of microbial contamination, but the condition of the cells does not appear to be compromised if the silicone fluid is not removed and the medium is not changed. If cells are cultured in bicarbonate-buffered media, then the medium will become alkaline in atmospheric CO₂. To solve this problem it is necessary to use a buffering agent such as HEPES, or to maintain elevated CO₂ concentrations on the microscope stage (McKenna and Wang, 1989).

Microinjection of adherent cells does require some practice, but once the technique is mastered, it is possible to routinely locate and inject 15 neurons in a single dish within 20 minutes. The principal challenges are to penetrate the nucleus with minimal damage to the cell, and to minimize blockage of the injector. To minimize blockages, we routinely clarify the injection solution by centrifugation or by filtration using an Amicon Ultrafree-MC 0.1 μ m centrifugal concentrator (Millipore Corporation). It is essential to include a fluorescent marker in the injection solution so that the flow rate can be adjusted empirically for each micropipette. The fluorescent marker also provides immediate visual confirmation of a successful injection (Fig. 2). We generally use rhodamine-labeled dextran (M_w 10,000 or M_w 70,000, Molecular Probes). The smaller dextran diffuses out of the nucleus within minutes after injection. To maximize cell survival, it is critical to use very low injection pressures. We typically use injection pressures of 7-14 hPa (0.1-0.2 psi) above the balance pressure (see below). Higher pressures result in damage to the nucleus. We have not measured the volume that is injected under these conditions, but it is small enough that we do not observe any noticeable increase in nuclear volume as judged by phase contrast or differential interference contrast microscopy. Too much pressure or too much volume (e.g. sufficient to cause a visible increase in nuclear volume) will result in cell death.

It is important to use a pressure injection apparatus that can supply a constant balance pressure to the injector at all times in order to prevent medium being drawn into the micropipette tip by capillary action between injections. The magnitude of the balance pressure should be adjusted empirically for each pipette so that injection solution is trickling out into the medium at all times (the fluorescent dextran in the injection solution allows the flow rate to be observed directly). Cells can be injected by initiating the injection pressure before penetration and terminating the injection pressure after the tip is removed

(“continuous flow technique”), or alternatively by initiating the injection pressure after the tip has penetrated the cell and terminating the injection pressure before it is removed. We generally use the latter method. The former method may reduce the chance of clogging during penetration, but it also results in more leakage of injection solution into the medium. This can temporarily obscure the fluorescence of the injected cell, making it difficult to determine whether the injection was successful.

In our studies using the GFP-NFM expression construct, about 20-30% of the injected cells typically survive and express GFP fluorescence, and the fusion protein becomes fully incorporated throughout the entire axonal neurofilament array within three days after microinjection (Wang *et al.*, 2000; Wang and Brown, 2001). The brightness of the GFP-fluorescence varies from cell to cell, depending on the expression level. Most of the cell death can be attributed to injury during the injection or toxicity due to over-expression of the GFP-NFM fusion protein. We have not investigated how soon GFP fluorescence first appears, but some delay is expected because GFP is known to acquire its fluorescent properties through a slow post-translational autocatalytic oxidative reaction that has been estimated to take several hours for wild type GFP (Heim *et al.*, 1994).

IV. Injecting neurons with fluorescent proteins

Slow axonal transport can also be studied by direct injection of fluorescently labeled protein into the cytoplasm of cultured neurons. For our studies on the axonal transport of microtubules, we injected rhodamine-labeled bovine brain tubulin (Cytoskeleton, Inc.) into the cytoplasm of cultured rat SCG neurons (Wang and Brown, 2002). The injection technique is similar to that describe above except that we pull micropipettes with slightly broader tips (lower velocity trip-point on the Sutter micropipette puller) and we use higher injection pressures. In general, cultured neurons will tolerate the injection of significant volumes into their cytoplasm and the survival rate is much higher than for nuclear injection, even if the volume injected is sufficient to cause the cell to swell visibly.

V. Observing movement

The study of slow axonal transport is challenging because of the small size and dynamic nature of the moving structures (macromolecular complexes of cytoskeletal and cytosolic proteins), and because of the rapid and intermittent nature of their movement. For example, observations on the slow axonal transport of cytoskeletal proteins require imaging strategies that are capable of detecting rapid, infrequent and asynchronous movements of cytoskeletal polymers (Brown, 2000). Here, we describe two such approaches that have enabled us to observe the rapid movement of neurofilaments and microtubules in axons of cultured SCG neurons.

A. Naturally occurring gaps

Cultured neurons from the SCG of neonatal rats express low levels of neurofilament protein and frequently exhibit short segments of axon that lack neurofilaments entirely. These “gaps” in the axonal neurofilament array arise naturally in these neurons and appear to have no functional significance, but they do present a unique opportunity for studies on the movement of neurofilaments in axons (Wang *et al.*, 2000; Roy *et al.*, 2000). To detect movement, a GFP-tagged neurofilament protein is expressed in cultured SCG neurons and allowed to incorporate throughout the endogenous neurofilament array, and then gaps in the axonal neurofilament array are located and observed by live-cell time-lapse fluorescence imaging. Rapidly moving neurofilaments can be detected as they move through these gaps because the gaps are devoid of other neurofilaments; essentially all of the neurofilament protein is polymerized in these neurons, so there is negligible background fluorescence due

to diffusible neurofilament subunits. We have also observed gaps in the neurofilament array of cultured rat brain cortical neurons, but not in cultures of rat dorsal root ganglion (DRG) neurons, which express much higher amounts of neurofilament protein. We have never observed gaps in the microtubule or microfilament array of any cultured neuron, and this is consistent with the fact that the continuity of these cytoskeletal arrays is critical for axon survival. Thus, while gaps are unique to the axonal neurofilament array, they are not unique to SCG neurons.

B. Photobleaching

Slow axonal transport can also be observed in cultured neurons using fluorescence photobleaching. For example, to detect the movement of neurofilaments, a GFP-tagged neurofilament protein is expressed in cultured SCG neurons and allowed to incorporate throughout the endogenous neurofilament array, and then the fluorescence is bleached in a segment of axon and the bleached region is observed by live-cell time-lapse fluorescence imaging. Neurofilaments that move into the bleached region are fluorescent (and can thus be detected) because they originate from the surrounding non-bleached regions of axon flanking the bleached region. In contrast to the observation of naturally occurring gaps in the neurofilament array, this approach can reveal the movement of neurofilaments in axons that do not exhibit gaps, and also the movement of other proteins that are distributed continuously along the axons. For example, we have recently used photobleaching to detect the movement of both GFP-tagged neurofilament protein and rhodamine-labeled tubulin in axons of cultured SCG neurons (Wang and Brown, 2001; Wang and Brown, 2002). By comparing the kinetics of neurofilament movement in photobleached axons and in naturally occurring gaps, we have established that the bleaching process does not impair the movement (Wang and Brown, 2001).

We perform the photobleaching using a standard Nikon TE300 or Nikon TE2000 inverted microscope equipped for epi-fluorescence and a Plan Apo 100x/1.4 NA phase contrast or DIC oil immersion objective. The region of axon to be bleached is positioned in the center of the field of view and then the field diaphragm in the epi-fluorescence illumination light path is closed down to illuminate only the area that is to be bleached. The fluorescence is then excited continuously for 30-120 seconds using a standard epi-fluorescence filter cube and epi-illumination from a standard mercury arc lamp (HBO100W/2). While the long duration of this bleaching exposure makes this approach unsuitable for many photobleaching applications, it is not a problem for studies on slow axonal transport because of the asynchronous and infrequent nature of the movement. To obtain the maximum possible light intensity for photobleaching, we remove all neutral density filters from the epi-fluorescence illumination light path for the bleaching exposure (radiant intensity = 600kW/m^2) and then replace them prior to subsequent imaging. Since bleaching at this intensity is relatively slow, it is desirable to select thin axons, which can be bleached more readily than thicker axons. Imaging can begin immediately after bleaching – no recovery period appears to be necessary. However, care must be taken to watch for evidence of photodamage caused by the bleaching process. We can bleach GFP-tagged neurofilament protein in axons of cultured SCG neurons for up to 2 minutes without noticeable impairment of neurofilament movement, so we believe that there is minimal photodamage under these conditions (Wang and Brown, 2001). Longer bleach times, however, can cause blebbing and vesiculation of the axons, which is a clear sign of damage.

Many previous studies on slow axonal transport in cultured neurons have used laser photobleaching, but those studies did not detect movement (Lim *et al.*, 1989; Lim *et al.*, 1990; Okabe and Hirokawa, 1993; Okabe and Hirokawa, 1990; Okabe *et al.*, 1993; Takeda *et al.*, 1994; Chang *et al.*, 1998; Takeda *et al.*, 1995; Sabry *et al.*, 1995). It now appears that those studies failed because they were designed with the expectation of a slow and

synchronous movement and were not capable of detecting rapid asynchronous movements (Brown, 2000). For example, those studies bleached short regions of axon (3-5 μm in length) and acquired images with long time lapse intervals (typically 5 minutes or more). Our photobleaching strategy differs from previous strategies in that it is designed with the explicit expectation of rapid asynchronous movements (Wang and Brown, 2001). The key features of our approach are: (1) the use of thin axons, which can be bleached sufficiently to allow the detection of the moving polymers without causing photodamage; (2) the use of short time-lapse intervals (2-5 seconds), which enables the detection of rapid movements (see Section V. C.); and (3) the use of long bleached regions (20-60 μm in length), which enables entire cytoskeletal polymers to be observed while they are moving.

Another difference between our photobleaching method and previous methods is that we use a mercury arc lamp to bleach the fluorescence. The principal advantage of this approach is that it can be performed with a standard epi-fluorescence microscope and that it is easy to bleach large areas of the field of view. However, a disadvantage of this method is that the relatively low energy of the mercury light source necessitates long bleaching times. More efficient bleaching could be accomplished using a laser, but lasers are not readily adapted to bleaching large areas of the field of view. A solution to this problem would be to bleach axons using a laser scanning confocal microscope, though we have not tried this ourselves.

C. Live-cell imaging

One of the challenges associated with live-cell fluorescence imaging is to minimize photobleaching and photodamage. Since photobleaching and photodamage are enhanced in the presence of oxygen, we observe the neurons in a sealed chamber containing oxygen-depleted medium (see below), essentially as described by Dent *et al.* (1999) (see Fig. 3). Cultured neurons thrive for many hours under these conditions because their energy metabolism is predominantly glycolytic. To assemble the chamber, we aspirate the medium from the culture dish (leaving medium in the well), rinse with deoxygenated culture medium, and then seal a cylindrical glass ring ("micro slide ring", 15 mm diameter, 5 mm high; Thomas Scientific) to the dish around the well using Type 111 silicone grease (Dow Chemical Co.). The chamber created by this ring is then filled to the brim with deoxygenated culture medium (volume = 0.9 ml) and a coverslip is sealed to the top of the ring using silicone grease.

To deplete oxygen from the culture medium, we use OxyraseTM (Oxyrase Inc.), which is a bacterial membrane fraction that contains enzymes of oxidative phosphorylation (Mikhailov and Gundersen, 1995). It is necessary to confirm the activity of the OxyraseTM in the culture medium (e.g. using an oxygen electrode) because it may not be as active as expected; while OxyraseTM can deplete oxygen from 50 mM phosphate buffer in a matter of minutes, we have found that it takes two hours in our L-15 based medium. Thus we incubate the culture medium with OxyraseTM at 37°C for at least 2 hours prior to assembling the imaging chamber. It is also critical to include adequate substrate for the OxyraseTM enzymes. We supplement the medium with 20 mM sodium succinate and 20 mM sodium DL-lactate (Sigma). We incubate the medium with the OxyraseTM in a disposable syringe, taking care to eliminate any air bubbles or spaces and to seal the opening with ParafilmTM. Since the membranes in the OxyraseTM tend to aggregate during the incubation, we attach a 0.2 μm syringe filter to the syringe and filter the oxygen-depleted medium directly into the imaging chamber. We then seal the chamber immediately so as to minimize exposure of the oxygen-depleted medium to the air. Care should be taken to avoid leaving any trapped air bubbles in the chamber as these will interfere with observation and possibly also compromise the efficacy of the OxyraseTM.

To maintain the temperature on the microscope stage, we use an air stream incubator (Nevtek). This approach has the advantage that it heats the dish, stage and objective. Cells thrive on the microscope stage for many hours with this arrangement. We usually warm the microscope for 30-60 minutes before use. Because the fan generates vibration, it must be isolated mechanically from the microscope. We place it on an equipment shelf supported above the vibration isolation table-top and direct the air stream under the stage of the inverted microscope so that it is angled up towards the base of the dish (in order to prevent vibration of the micropipette during microinjection).

To observe the cells, we use a Nikon TE300 or TE2000 inverted microscope. For fluorescence imaging, we try to use optics with the highest numerical aperture and light throughput. In our experience, Plan Apo DIC objectives are generally the best choice. We recommend that individual objectives be compared quantitatively because we have found significant differences that would not be apparent from the manufacturer's specifications. Fluorescent polystyrene beads (Molecular Probes) that fluoresce across the entire visible spectrum are excellent for such quantitative comparisons because they are bright, uniform and very photostable. It is also desirable to use the most efficient light path on the microscope (minimizing any lens elements that might lead to loss of light) and to use the most sensitive detection device available.

In selecting a camera for live-cell fluorescence imaging, three features are of paramount importance: sensitivity, resolution and speed. Generally there is a trade-off between speed and resolution on the one hand and sensitivity on the other. According to the Nyquist theorem, the best possible resolution is obtained when the pixel size of the camera chip is less than half the optical resolution of the microscope objective (Young, 1989). We are currently using two different cooled CCD cameras for live-cell imaging. For better sensitivity, we use a Princeton Instruments Micromax 512 BFT camera with 512×512 back-illuminated chip (Roper Scientific). The pixel size of this chip is $13.0 \times 13.0 \mu\text{m}$, which corresponds to $0.131 \mu\text{m}$ per pixel using a 100x objective on our inverted Nikon microscopes. This camera has a full frame readout time of 350 msec, a quantum efficiency of about 85% in green light, and a read noise of 8 electrons at 1 MHz. For better speed, we use a Photometrics CoolSNAP HQ camera with 1392×1040 front-illuminated interline chip (Roper Scientific). The pixel size of this chip is $6.45 \times 6.45 \mu\text{m}$, which corresponds to $0.065 \mu\text{m/pixel}$ when using a 100x objective on our inverted Nikon microscopes. This camera has a full frame readout time of 100 msec, a quantum efficiency of about 60% in green light, and a read noise of 6 electrons at 10 MHz. Since our exposure times tend to be quite long (typically 1 second), we have found that the sensitivity of the camera (determined by quantum efficiency and read noise) can sometimes be a more critical determinant of the rate of image acquisition than the actual readout time. In other words, a camera with a shorter readout time may not necessarily result in faster image acquisition if longer exposures and/or frame averaging are required to detect the fluorescent signal.

To observe movement, fluorescent cells are located at low magnification using a 10x or 40x objective and then examined at high magnification using a Plan Apo 100x/1.4 NA DIC or phase contrast oil immersion objective. We avoid cells that are overly bright because of the potential for artifacts associated with high concentrations of exogenous protein. To find gaps in the neurofilament array or to locate axons that are suitable for photobleaching, we scan along the axons using epi-fluorescence and either phase contrast or DIC, always taking care to observe the fluorescence as little as possible. We stop down the field diaphragm in the epi-fluorescence illumination light path to prevent exposing areas outside the field of view. The neurons are cultured at low density (see Section II. A.) so that axons can be traced from the cell body to the growth cone. This is necessary in order to establish the proximal and

distal orientation of the axons in the time-lapse movies. The low density also helps to minimize axonal fasciculation.

Time-lapse fluorescence images are acquired using the “Acquire Time-lapse” function in Metamorph™ software (Universal Imaging Corporation). We use electronic shutters on the mercury and halogen light sources, which can be controlled with foot switches (for hands free manual operation) and from the Metamorph™ software (for automated operation during image acquisition). To reduce photobleaching, it is necessary to minimize the intensity of the exciting light and the duration of the fluorescence exposures. Typically we attenuate the mercury light source by 10-15% using neutral density filters and use exposures of about 1 second. Under these conditions, we are typically able to acquire time-lapse movies that are 100-200 images in length. Since the movements are very infrequent, it is not unusual to acquire movies in which no movement is observed, especially if the movie is short. For example, in our studies on neurofilaments we observed an average of one moving neurofilament every 5 minutes (Wang and Brown, 2001; Wang *et al.*, 2000). To maximize the duration of the movies, we use the longest time-lapse intervals that we can without sacrificing our ability to track the movements. For studies on neurofilaments, time-lapse intervals of 4 or 5 seconds are a good compromise, allowing us to acquire movies that are approximately 5-15 minutes in length. The principal factors limiting the duration of our time-lapse movies are the sensitivity of the camera and the brightness and photostability of the GFP.

A problem that we often encounter during the acquisition of time-lapse movies is focus drift. The causes of focus drift are either mechanical (e.g. instability in the focusing mechanism of the microscope) or thermal (expansion and contraction of the microscope stage and/or optics). With practice, one can learn to anticipate and correct for this drift during the image acquisition by making small adjustments to the fine focus on the microscope in between image acquisitions. We have found that a motorized Z-axis (Prior Scientific) helps by giving the user more precise control over the fine focus movement. It is also helpful to keep the temperature of the microscope and room as stable as possible.

D. Motion analysis

The movement of fluorescent structures can be tracked in successive frames of the time-lapse movies using the “Track Points” function in the Metamorph™ software and the data can be logged directly to an Excel worksheet. It is important to establish objective criteria for the analysis. We generally exclude structures if they move less than a total distance of 10 μm during the course of the movie or if their location is ambiguous: this excludes objects that jiggle around but do not display directed movement. Ambiguity can arise due to poor focus, overlap with other moving or pausing structures, or loss of brightness due to photobleaching. When tracking the movement of neurofilaments or microtubules, we generally follow the position of the leading end. Fiduciary points in the field of view can be used to ensure that there is no stage drift during the movie, though this is rarely a problem. If stage drift does occur, the planes in the stack can be registered using the “Align Stack” function in Metamorph™. For presentation, we save the time-lapse images in RGB tiff format using the “Convert Stack to TIFFs” feature in the Metamorph™ software, and then import them into Adobe Premiere for movie construction and editing. QuickTime movies are created with a play-back rate of 6-8 frames per second. If necessary, the movies can be compressed with a lossy compression algorithm (“codec”) such as Cinepak.

To facilitate detection of the moving structures in the time-lapse movies, we sometimes perform difference imaging. To obtain a rolling difference image, each image in the time-lapse series is subtracted from the image that follows it. To obtain a fixed-frame difference image, a single reference image at the start of the time-lapse series is subtracted from all

subsequent images in the series. Both methods selectively enhance changes in fluorescence intensity within the field of view, such as those associated with the movement of fluorescent structures. We have found difference imaging to be especially useful for our photobleaching studies on microtubule movement in axons because the pool of unpolymerized tubulin rapidly recovers its fluorescence after bleaching due to the diffusion of fluorescent tubulin subunits from the adjacent unbleached regions, and this fluorescence is often sufficient to completely or partially obscure the moving polymers in the raw images (Wang and Brown, 2002).

VI. Conclusion

For many years it has been assumed that the proteins conveyed by slow axonal transport move in a slow and synchronous manner, but attempts to observe this movement by live-cell imaging have been largely unsuccessful. Recent observations by ourselves and others on the movement of neurofilaments and microtubules in axons of cultured neurons have demonstrated that the movements are neither slow nor synchronous; both neurofilaments and microtubules actually move in a rapid and asynchronous manner, but the overall rate is slow because the movements are also very infrequent. In other words, these structures spend most of their time not moving during their journey down the axon. This remarkable motile behavior presents special challenges for live-cell imaging studies, but I hope that this chapter has demonstrated that they are not insurmountable and that movement can be detected if the experiments are designed with the expectation of rapid and infrequent movements.

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Abbreviations

SCG	(superior cervical ganglion)
DRG	(dorsal root ganglion)
GFP	(green fluorescent protein)
TE	(Tris/EDTA buffer)
DIC	(differential interference contrast)

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Fig. 1. The coverslip culture dish assembly

A hole is drilled in the bottom of a 35 mm culture dish and a glass coverslip is affixed to the base of the dish using paraffin wax. The cells are plated onto the coverslip in the shallow well created by the hole in the bottom of the dish.

Fig. 2. Nucleus injected with fluorescent dextran

Cell body of a cultured SCG neuron imaged by epi-fluorescence microscopy immediately after microinjection of the nucleus with M_w 70,000 rhodamine-labeled dextran. The dark spot within the nucleus represents the nucleolus, which excludes the fluorescent dextran. Scale bar: 10 μ m.

Fig. 3. Sealed imaging chamber for live-cell fluorescence imaging

The chamber is constructed by placing a cylindrical glass ring around the well, filling it with oxygen-depleted medium, and then covering it with a glass coverslip. The glass ring is sealed to the dish and to the top coverslip using silicone grease.

Fig. 4. Movement of a neurofilament through the photobleached region of an axon
Axon of a cultured SCG neuron expressing GFP-tagged neurofilament protein. These images were selected from a time-lapse movie in which images were acquired at 4-s intervals. GFP fluorescence was bleached as described in Section V,B. A fluorescent neurofilament can be seen moving in an anterograde direction through the photobleached region. Small white arrowheads mark the leading and trailing ends of the moving neurofilament, and large white arrowheads mark the edges of the photobleached region. Proximal is left and distal is right. Data from Wang and Brown (2001). Scale bar: 5 μm .