c and other proteins from the intermembrane space may arise from the complexity of events that impinge on and overlap with each other at the mitochondrial level during cell death. Goldstein *et al.*'s experiments[1] offer an example of how it is possible to dissect and order some of these mitochondrial events. The use of cell-free systems may also be helpful, in particular in the understanding of how pro-apoptotic members of the Bcl-2 family can trigger outer-membrane permeability specifically. A more precise understanding of these mechanisms should allow the identification of therapeutic targets for the development of drugs that will modulate the cell death associated with many pathological states. □

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Neurofilaments run sprints not marathons

Scott T. Brady

In neurons, cytoskeletal proteins are transported from where they are made — the cell body — along the axons, but it has long been disputed whether they are transported as subunits or polymers. A new analysis of neurofilament movement may help to resolve the controversy.

An often-overlooked aspect of neurons is their size. In humans, many neurons extend cytoplasmic processes, or axons, of a metre or more in length, making these neurons the largest cells in the body. Remarkably, synthesis of all axonal proteins, including components of the cytoskeleton, is restricted to a cell body a few tens of micrometres in diameter. Every protein has to be transported from where it is made to where it is needed. Pulse-labelling studies indicate that cytoskeletal proteins move away from the cell body at an average rate of a millimetre per day. As a result, cytoskeletal proteins in long axons spend years in transit. However, when Wang and colleagues — writing on page 137 of this issue[1] — directly observed movements of a neurofilament subunit fused to a fluorescent marker protein, they found that individual neurofilaments can move a hundred times faster than this average rate.

This observation is the latest chapter in a longstanding controversy over the molecular mechanisms for delivery of cytoskeletal elements to axons. Two schools of thought have framed the debate. One school emphasizes the movement of structures corresponding to assembled microtubules or neurofilaments, arguing that assembly of microtubule or neurofilament subunits into polymers is necessary for the transport of these subunits. This is generally known as the 'structural hypothesis'. Although early formulations of this model proposed a continuous, crosslinked microtubule–neurofilament network moving synchronously down the axon, this idea soon gave way to a model in which individual neurofilaments and microtubules move more or less independently[2].

The other school of thought holds that cytoskeletal structures such as microtubules and neurofilaments form a crosslinked network that is essentially non-motile. In this model, individual subunits or small oligomers distinct from conventional cytoskeletal structures move down the axon. These subunits then exchange with subunits of stationary cytoskeletal elements, to allow turnover of cytoskeletal proteins in the axon. This theory has sometimes been called the 'subunit hypothesis'[3].

Over the years, proponents of each hypothesis have cited experiments that they believed were compelling, but neither faction managed to convert the other side. Comparable disputes once surrounded the mechanisms of vesicle transport, but many of the questions were answered once methods for visualization of vesicle movements in living cells became available[4]. Unfortunately, methods for visualizing movement of cytoskeletal materials have not existed until recently.

The first opportunity to follow the movement of cytoskeletal proteins in living neurons or neuron-like cells in culture came with the addition of fluorescent tags to cytoskeletal proteins. Fluorescently tagged cytoskeletal proteins, microinjected into neurons, typically filled a neurite. A patch on the neurite could be 'photobleached' and monitored. One early experiment showed a slow anterograde movement of the bleached spot at a rate comparable to that revealed by pulse-labelling studies, as predicted by the structural hypothesis[5]. However, other researchers, using slightly different procedures, found that the bleached spot remained stationary, gradually recovering its fluorescence without obvious directionality (see ref. 3 for a review). These results seemed more consistent with the subunit hypothesis. When photoactivation of a caged fluorophore on tubulin (the microtubule subunit) was used to minimize photodamage, a fluorescent patch that exhibited slow anterograde movement was seen[6]. However, subsequent studies saw movements in *Xenopus* but not mouse neurites[7]. Recently, when lower levels of fluorescent tubulin were injected into neurons, producing speckled microtubules, axonal microtubules appeared stationary[8], but concurrent studies described movement of individual microtubules at axonal branch points and growth cones[9]. Injection of fluo-

...Remarkably, synthesis of all axonal proteins is restricted to a cell body a few tens of micrometres in diameter. Every protein has to be transported from where it is made to where it is needed.....

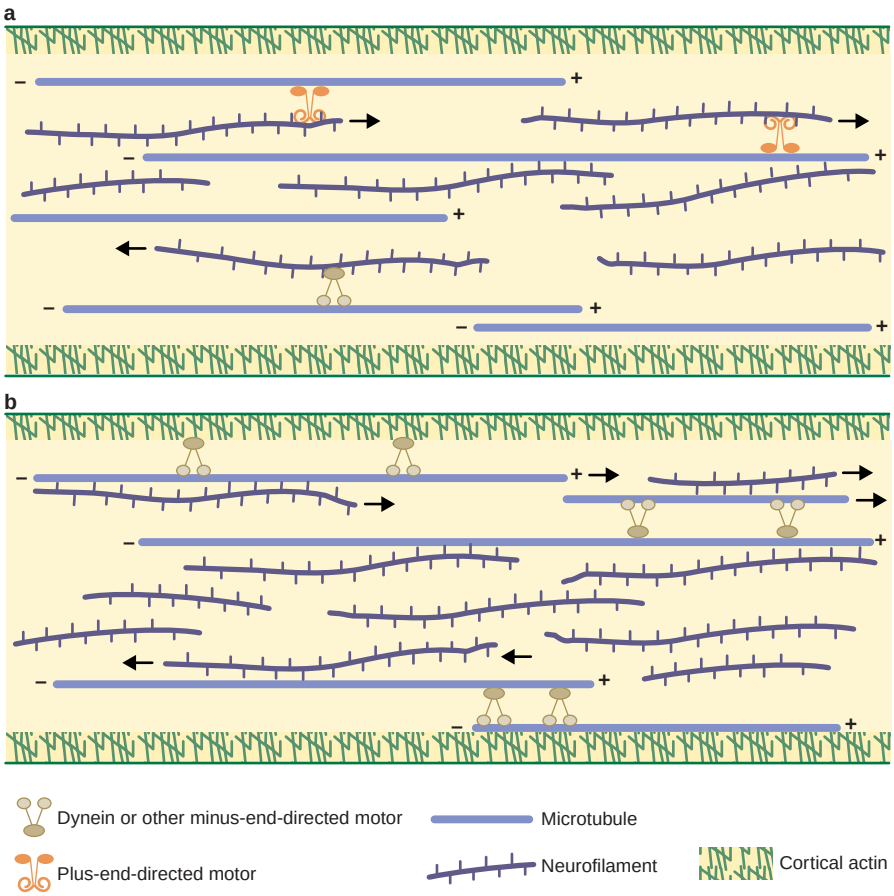

Figure 1 **Two models for neurofilament movement along neuronal axons.** a, **In this model, neurofilaments bind to stationary microtubules through microtubule-associated motor proteins (such as dynein or other motors that move towards the so-called 'minus' ends of microtubules, or kinesin or other plus-end-directed motors). The motors then 'walk' along the stationary microtubules, carrying their cargo (the neurofilaments) with them along the axon.** b, **In this model, the neurofilaments bind directly to microtubules. The microtubules themselves are transported along the axon and the neurofilaments are moved with them. Arrows indicate the direction of microtubule/neurofilament movement.**

rescently tagged cytoskeletal proteins failed to resolve the issue.

The ability to express recombinant intermediate-filament proteins fused to green fluorescent protein (GFP) represents the newest approach to the problem. When GFP-tagged vimentin was expressed in cultured cells, short filamentous structures and 'dots' of GFP could be visualized moving in multiple rates and directions[10]. However, the distances travelled by vimentin in epithelial cells are relatively short. Moreover, vimentin filaments are more labile than neurofilaments and neurofilaments rapidly polymerize *in vivo*[11].

Wang *et al.*[1] have now microinjected complementary DNA encoding GFP-tagged neurofilament protein M (GFP–NFM) into cultured sympathetic neurons, which have low levels of endogenous neurofilament protein. Fluorescence from GFP–NFM appeared in neurites as a discontinuous pattern, enabling visualization of individual filaments. By several criteria, including the filamentous appearance of the NFM–GFP, its insolubility, and its localization together with low-molecular-mass neurofilament subunits, Wang *et al.* judged that the GFP–NFM had assembled into neurofilaments. Even small 'dots' of GFP–NFM were likely to represent neurofilaments containing patches of GFP–NFM, given that these dots co-localized with antibodies that label neurofilaments. In a given neurite, GFP–NFM-labelled neurofilaments could be seen to move at a speed of several micrometres per second. However, motile GFP–NFM frequently paused, and much axonal GFP–NFM did not move during the course of an experiment. Based on the observed rates and estimated fraction of time spent moving, the average speed of GFP–NFM appears consistent with transport at roughly a millimetre per day. Neurofilaments were not moving most of the time, although a small percentage of neurofilaments moved rapidly along the axon.

Why weren't these rapid movements seen in earlier studies? In part, there were limitations in the methods or experimental design. In the pulse-labelling studies, for example, rates of movement represent an average velocity for the bulk of the labelled neurofilaments. Typically, the time points studied are days or weeks after labelling, when rapidly moving fractions are long gone. One study that looked at shorter time intervals found that a small fraction of total labelled neurofilaments did move rapidly[12], but the temporal resolution and sensitivity of pulse-labelling studies are generally ill-suited to detect fast neurofilament movements.

Similarly, in photobleaching and photoactivation experiments researchers looked for slow synchronous movement of neurofilaments. In these experiments, time-lapse video microscopy was used to analyse cytoskeletal movements, with long intervals between frames (minutes or hours) being important to preserve fluorescence intensity. Rapid movement of fluorescent neurofilaments (or microtubules) through a photobleached segment would rarely be captured at this sampling rate, as shown amply now by Wang *et al.*[1]: in their experiments, neurofilament movements were not synchronous and were best visualized by capturing frames at five-second intervals. Earlier experiments in which synchronous slow movement was observed may reflect culture conditions that enhance synchrony of neurofilament transport.

What does all this tell us about how neurofilaments move? There is a general consensus that microtubules are required for neurofilament transport, but less agreement about how transport actually occurs. From Wang *et al.*'s data, two models of movement emerge (Fig. 1). In the first model, neurofilaments move in ways analogous to the movement of vesicles along microtubules (Fig. 1a). One or more microtubule-associated motor proteins associate with neurofilaments, and carry their cargo along stationary microtubules. In this model, pauses might be the result of transient interactions between motors and neurofilaments. Consistent with this model, Goldman and colleagues reported that the microtubule motor kinesin was associated with vimentin-containing structures[13], as judged by the patterns of antibodies that identify these two proteins. However, the patterns of co-localization of vimentin-containing structures and kinesin do not match distributions seen with other anti-kinesin antibodies, and in another study no kinesin was detected moving with neurofilaments along the axon[14].

The second model does not require direct interaction between motors and neurofilaments. Instead, neurofilaments attach directly to microtubules (Fig. 1b). When the microtubule is moved by a dynein- or kinesin-related motor protein, attached neurofilaments are carried along as hitchhikers. This model is consistent with the extensive interactions between microtubules and neurofilaments seen in electron micrographs. Many kinesin-related proteins and dyneins bind to one microtubule while moving along a second, although no direct evidence exists for concurrent movement of a microtubule and a neurofilament. However, neurofilaments do accumulate in initial segments of axons together with some neurotoxins, and in neuropathologies such as amyotrophic lateral sclerosis, even while microtubule proteins continue into the axon. The reverse situation has not been observed, indicating that neurofilaments may need moving microtubules.

We cannot distinguish between these two models yet. Nonetheless, Wang *et al.* show clearly that neurofilaments can and do move down axons[1]. The high rates of movement and long pauses between movements of axonal neurofilaments that Wang *et al.* observed help to reconcile many apparently contradictory results. Experiments such as these provide further insight into how cytoskeletal elements can both move down an axon and provide structural support for that axon at the same time. □

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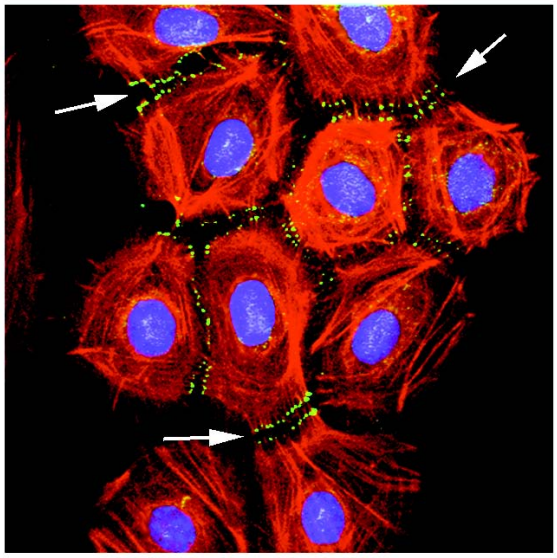
Zipping up adhesion

Cell–cell adhesion is crucial during epithelial development and morphogenesis, with adherens junctions being the main intercellular structures. At each adherens junction, the calcium-dependent cell-adhesion molecule E-cadherin is directly linked to the actin cytoskeleton through α- and β-catenin. Other cytoskeletal proteins, including vinculin, α-actinin and VASP, have also been implicated, either directly or indirectly, as being part of the adherens-junction structure. During adherens-junction formation, E-cadherin initially localizes to 'punta', formed at the first sites of cell–cell contact. Time-lapse imaging and electron-micrographic studies have revealed a subsequent rapid reorganization of the cytoskeletal actin filaments protruding from these puncta, possibly mediated through the GTPase Rho, leading to closure and sealing of the two opposing cell membranes.

Valeri Vasioukhin and colleagues (*Cell* **100,** 209–219; 2000) have now shown that the driving force behind E-cadherin-mediated puncta formation, and rapid actin polymerization, is an increase in calcium levels. Vasioukhin *et al.* found that, three hours after primary epithelial keratinocytes are moved from low to high levels of extracellular calcium, filopodia — cellular projections — are formed. These filopodia first penetrate, and then embed into, their neighbouring cells. Two opposing rows of E-cadherin (green in the figure opposite) are then seen at these points of contact in punctate pre-adherens junctions. (Arrows in the figure point to so-called adhesion 'zippers'.) Once the puncta are formed, rapid actin polymerization and cytoksleletal reorganization act to pull the opposing membranes together, 'zipping' up the membrane. Desmosomes — structures containing cadherin-family members linked to the intermediate-filament network — then clamp opposing cell membranes at areas unoccupied by adherens junctions. Sealing of the opposing membranes follows.

Vasioukhin *et al.* go on to show that VASP and MENA, a VASP-family member, are essential for membrane zippering. In cultures of α-catenin-deficient keratinocyte cells, there are no E-cadherin zippers, no actin polymerization and, most interestingly, no localization of MENA/VASP to the punctate adherens-junction structures. The



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authors therefore propose that α-catenin acts to recruit these proteins to the adherens junction, and that it is these proteins, together with vinculin and zyxin, that provide the physical force for rapid actin polymerization and membrane zippering.

It appears that cell–cell adhesion is far from being a 'stick' and 'attach' process: complex interactions between different cytoskeletal proteins are being uncovered. Furthermore, as E-cadherin is a tumour-suppressor protein, loss-of-function mutations in which generate diminished cell–cell adhesion and increased cellular motility, future work should allow us to understand the importance of adhesion regulation and its implications in cellular transformation.

SARAH GREAVES

