The evolution of the axonal transport toolkit

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Synopsis
Long-range axonal transport is critical for neuronal homeostasis and function. Since its discovery, advances in its understanding have been aided by scientific discoveries in other disciplines, as well as the development of novel tools, techniques and model systems. In this review, we discuss the evolution of the tools used to investigate axonal transport and how they have furthered our understanding of this process. Furthermore, we highlight innovative probes that could potentially be added to this ever-expanding toolkit.

Abstract
Neurons are highly polarised cells that critically depend on long-range, bidirectional transport between the cell body and synapse for their function. This continual and highly coordinated trafficking process, which takes place *via* the axon, has fascinated researchers since the early twentieth century. Ramon y Cajal first proposed the existence of axonal trafficking of biological material after observing that dissociation of the axon from the cell body led to neuronal degeneration. Since these first indirect observations, the field has come a long way in its understanding of this fundamental process. However, these advances in our knowledge have been aided by breakthroughs in other scientific disciplines, as well as the parallel development of novel tools, techniques and model systems. In this review, we summarise the evolution of tools used to study axonal transport and discuss how their deployment has refined our understanding of this process. We also highlight innovative tools currently being developed and how their addition to the available axonal transport toolkit might help to address key outstanding questions.

Introduction
The precise subcellular spatial organisation of organelles, proteins and RNAs is critical in maintaining cellular function and homeostasis. Such precise organellar and molecular distribution is particularly challenging in neurons, given their unique morphology and high degree of polarisation, where, in the most extreme examples (e.g. motor and sensory neurons), axons can reach lengths of five orders of magnitude that of the cell body. Neurons achieve this complexity through a process called axonal transport, whereby motor proteins actively traverse tracks of polarised microtubules along the axon to deliver diverse cargoes to the synapse[1]. Anterograde transport from the cell body to axon terminals, which is mediated by the kinesin family of molecular motors, traffics essential proteins, RNAs and organelles such as mitochondria[2]. On the other hand, axonal retrograde transport, driven by cytoplasmic dynein, connects the synapse to the cell soma and is required for the delivery of survival signals from the cell periphery, as well as degradation and recycling of superfluous and damaged proteins and organelles[3]. Based on their velocities, trafficking cargoes are characterised as employing fast (~100-400 mm/day) or slow (~0.1-4 mm/day) axonal transport modalities. Lysosomes, mitochondria, presynaptic components and mRNAs undergo fast transport, whereas cytosolic proteins and cytoskeletal components exhibit slow axonal movement[4]. The importance of axonal transport in neuronal homeostasis is underscored by observations that deficits in this process are directly linked to many neurological diseases[5]. Unsurprisingly, the intricacies of neuronal trafficking have fascinated researchers since the early twentieth century.

One of the pioneers in neuroscience research, whose work suggested the possible existence of an axonal transport system, was Santiago Ramon y Cajal. After observing that separation of neuronal cell bodies from their axons resulted in neurodegeneration, Cajal concluded that the cell body, acting as the trophic centre of the neuron, sends out growth and survival signals to the axon[6]. However, it was not until the work of Paul A. Weiss and Helen Hiscoe published in 1948 that axonal transport, first termed 'axoplasmic flow', was observed experimentally[7]. By constricting tibial and peroneal nerves of rats, which had been crushed to instigate nerve regeneration, Weiss and Hiscoe observed a bulging of the nerves proximal to the blockade that resulted in a steady proximal-to-distal wave-like flow of material once the constriction was removed. This peristaltic 'flood wave' advanced anterogradely at a rate akin to the speed of regenerating nerve outgrowth, and was the first direct evidence for axonal transport.

Yet it would take another 15 years or so and the work of several other groups before this pioneering study garnered wide acceptance[8]. Much of this delay reflects the technical difficulties associated with studying a dynamic process in experimentally intractable, electrically excitable cells. As such, the history of advances in the field very much coincides with, and has been driven by, the evolution of tools necessary to study axonal transport[9] as well as discoveries in other scientific disciplines. Over the last 30 years, the implementation of visualisation tools and techniques to observe individual molecular cargoes being trafficked along axons, at first *in vitro* and now more frequently *in vivo*[10,11], has rapidly increased the rate of discovery in axonal transport.

For this special 20th anniversary Platinum Issue of Traffic, we will provide a perspective on the evolution of tools to study fast axonal trafficking, highlighting major breakthroughs and outlining how the development of novel tools has shaped and refined our understanding of axonal transport. We will begin by discussing techniques that helped to identify the fundamental nature of transport along axons, and continue by covering experiments that started to reveal the identity of molecular players that act as drivers and cargoes of axonal trafficking. We will then discuss tools and techniques that have shed light on the dynamics and regulation of fast axonal transport, including its coordination in response to external and internal cues. We will conclude by outlining novel tools that can now be exploited to address key questions in the field, thus furthering our knowledge of this fundamental process.

What is the nature of transport between the neuronal cell body and axon terminal?

After Cajal's seminal hypothesis in the early twentieth century suggesting the existence of an axonal transport system between the cell body and axon[6], treatment of limb injuries sustained by soldiers in World War II revived an interest in nerve growth and regeneration. Studies into axonal transport were initiated due to a desire to understand how the volume of the axonal cytoplasm is maintained in mature neurons and restored in regenerating neurons; that is, axonal transport was first studied as a question pertaining to cellular growth[7]. In this context, Weiss and Hiscoe, using nerve ligations on rats and classical histochemistry, observed that accumulated material around the constriction moved in a proximo-distal direction at a velocity of 1-2 mm per day upon removal of the constriction[7]. This was the first description of 'slow transport' in mature neurons and was thought, for the next decade, to be the main mode of axonal trafficking. Following this finding, metabolic labelling and pulse chase experiments, in addition to ligations, were used to gain quantitative insights into the axonal transport of proteins[12], phospholipids[13], RNA[14], glycoproteins[15] and glycolipids[16]. *In vivo*, a radiolabelled precursor was injected close to the neuronal cell body, which, upon incorporation into newly synthesized molecules, would be transported into the axon. *In vitro*, cell bodies of isolated neurons would be placed in media containing the radioactive tracer, while axonal termini would be immersed in non-radioactive media, thus enabling localised labelling. Segments of these axons collected at different time points post-injection were then subjected to either electron microscopy and autoradiography or cellular fractionation to quantify rates of axonal trafficking[17] (**Figure 1**, red box). Using [^{3}H]-leucine injections in sciatic nerves of rats followed by autoradiography, Droz and Leblond directly confirmed the existence of slow transport[18]. Similar experiments employing radiolabelled leucine injections in the dorsal root ganglia (DRG) of adult cats allowed the visualisation of anterogradely moving axonal components with 'fast' transport speeds (500 mm/day), in addition to compartments moving with at least three intermediate speeds[19]. Subcellular fractionation of

these labelled sensory axons firmly established the presence of fast and slow axonal trafficking in neurons[20], twenty years after Weiss and Hiscoe first reported this phenomenon.

Despite gaining wide acceptance due to its sensitivity and *in vitro* and *in vivo* applicability, radioactive labelling suffered from two serious drawbacks: i) distinguishing between transported, free precursor and incorporated precursor was difficult; and, ii) precursors would often get incorporated into neighboring cells near the site of injection, leading to background radioactivity. To overcome these limitations, Fink and Gainer pioneered *in situ* covalent labelling of axonal proteins using membrane-permeant, radioactive [2,3-^{3}H]N-succinimidyl propionate, which could then be investigated using autoradiography and gel electrophoresis[21]. Researchers also started exploiting trafficked enzymes[22] and neurotransmitters[23] to trace axonal transport. While neurotransmitters were followed by radiolabelling, spatiotemporal accumulation of enzymes was studied by exploiting histochemical reactions catalyzed by the enzyme under consideration. After observing that the enzyme acetylcholinesterase accumulates on both sides of a nerve ligation, Lubinska and colleagues suggested that anterograde transport from the cell body to axon terminal is accompanied by retrograde movement towards the cell body[24,25]. The occurrence of retrogradely-moving molecules had previously only been inferred, based on the observation that several neurotropic viruses and bacterial toxins gain an entry into neurons after targeting their distal axonal ends[26]. Radiolabelling supported the existence of bidirectional axonal transport[27] and was confirmed by direct light microscopy visualization[26]. Cessation of acetylcholinesterase transport by treatment with the microtubule inhibitor colchicine in rat sciatic nerves also established microtubules as the tracks on which axonal trafficking occurs[28]. Each of these labelling and manipulation methods that led to the discovery of bidirectional axonal transport were initial attempts to study this process in a gamut of neuronal cell types and models and set the stage for the array of tools and techniques that the field currently employs.

Cargoes and molecular drivers of fast axonal transport

After establishing fast axonal transport as a biological phenomenon, the identity of the molecules and organelles transported remained a mystery for a long time. The discovery of retrograde trafficking and limitations of metabolic labelling spurred the initial use of exogenous tracers, such as Evans blue-albumin conjugates[29] and horseradish peroxidase (HRP)[30] as neuroanatomical tracers. These molecules could be applied to axonal termini and, after uptake and retrograde transport, would label the axon as well as the cell body, thus bypassing the blood-brain barrier[17,31]. HRP, in particular, became a widely used tracer because it could be used *in vitro* and *in vivo* with high sensitivity, and the histochemical reaction with its substrate 3,3'-diaminobenzidine was compatible with light and electron microscopy[30,31]. Experiments with HRP suggested the involvement of retrograde transport in target tissue signalling[32] as well as in survival signalling during injury and regeneration in neurons[31].

Despite its widespread use, HRP was not exempt from complications. The granularity of its reaction product often failed to label the entire neuronal cell body, whilst its uptake, largely non-specific, required the application of high concentrations of tracer[33]. Additionally, the identity of the compartments transporting HRP was unclear[31]. To circumvent the lack of specificity in HRP endocytosis, plant lectins, which bind to glycoproteins and glycolipids[34], emerged as alternative neuronal tracers[35,36]. Radiolabelled or HRP-conjugated lectins were employed for tracing anterograde and retrograde transport *in vivo*[35,37–39]. The most frequently used lectin was wheat germ agglutinin (WGA), which binds to N-acetyl-glucosamine and sialic acid with high affinity.

Radiolabelled WGA is retrogradely transported in all peripheral neurons[40], as well as anterogradely in chick retinal ganglion cells[41]. The uptake of WGA-HRP *in vivo* allowed its accumulation in cisternae, vesicles and other intracellular membranes[42,43].

The need of an histochemical assay to visualise HRP limited the development of novel HRP conjugates, and at the same time drove the development of fluorescent tracers for axonal transport[44]. The lipophilic carbocyanine dyes DiI and DiO, despite being distributed along the axon by lateral diffusion on the plasma membrane rather than undergoing active axonal transport[45], proved to be efficient anterograde and retrograde tracers[46]. On the other hand, the membrane-permeant tracer Fluorogold is retrogradely transported *in vivo* in acidic compartments, is compatible with immunohistochemistry and electron microscopy and is readily quantifiable by spectrometry[47–50]. Other fluorescent tracers, such as Lucifer yellow, Texas red and Lissamine Rhodamine B sulfonyl chloride were used to study retrograde transport in mice corticospinal tracks[51]. These fluorescent tracers allowed the observation of autophagic vesicles and newly-formed endosomes and their retrograde transport *in vitro*[52].

The binding of lectins to glycosylated proteins on the plasma membrane was a useful tool to trace the transport of ligand-dependent endocytosis; however, the identity of the proteins transported remained largely unclear. Therefore, the uptake and retrograde transport of monoclonal antibodies directed to proteins of the synaptic membrane was implemented[53,54], spurred by discoveries describing the molecular composition of the synapse[55]. Radiolabelled antibodies against dopamine β-hydroxylase had previously been used in sympathetic neurons *in vivo*[56], opening up the possibility of studying the trafficking of individual proteins. One of these was nerve growth factor (NGF), a member of the neurotrophin family. Neurotrophins are target tissue-derived growth factors that regulate neuronal growth, survival and maintenance. NGF was the first neurotrophin discovered[57,58], with brain-derived neurotrophic factor (BDNF), neurotrophin-3 and -4 completing the family[59]. The retrograde transport of [^{125}I]-NGF was described in sympathetic neurons *in vivo*[60], while antibodies against the low affinity neurotrophin receptor p75^{NTR} were also used[61]. These antibodies were crucial to differentially manipulate the retrograde transport of neurotrophins[62] and to pinpoint the kinetics and downstream effectors of neurotrophin receptors[63], linking their axonal transport and signalling pathways.

The toolbox for studying transport of individual cargoes along the axon was not limited to endogenous neuronal proteins and their differential labelling methods. Neurotropic viruses and bacterial toxins, which had initially indicated the existence of retrograde trafficking, have also been used for tracking axonal transport. For example, tetanus neurotoxin (TeNT) is retrogradely transported in adrenergic, sensory and motor neurons in the peripheral nervous system[64]. Initial experiments to study its transport and subsequent pathogenicity relied on applying the full-length toxin to the mammalian neuromuscular junction (NMJ)[64]. However, biochemical studies later indicated that TeNT is formed by a heavy chain and light chain held together by a disulphide bridge, with the intracellular toxic activity located in the light chain, whilst the heavy chain is responsible for TeNT internalisation[65,66]. In fact, papain-mediated proteolysis of TeNT generates a heavy chain C-terminal fragment (H$_C$T) that is sufficient for membrane binding, uptake and retrograde transport[67–69]. This atoxic fragment can be used for the delivery of covalently coupled enzymes[70,71] and for studying intracellular trafficking routes (discussed in the next section). H$_C$T was also found to share axonal retrograde carriers with NGF and BDNF and can, therefore, be used as a reporter to trace organelles carrying neurotrophic signals[72,73]. Cholera toxin is another

bacterial protein toxin transported along the axon[40,74]. Structurally, it is a hetero-hexamer composed of one active A subunit and five B subunits (CTB), the latter being necessary for its internalisation and transport. As a result, several studies have used fluorescent CTB as a bidirectional tracer and as a marker for retrogradely transported endosomes[75–77]. For viruses, such as herpes simplex virus and poliovirus, the focus has been on studying their hijacking of axonal transport mechanisms for their pathogenicity, as opposed to their utility to study trafficking[78,79]. Nevertheless, the many different strategies for their efficient propagation across the nervous system makes them promising tools to shed light on additional aspects of axonal transport.

The neuronal probes described so far were useful for the characterisation of proteins and organelles being trafficked along the axon. However, to determine the molecular drivers responsible for this transport, a different approach was taken. Two developments made this discovery possible: an improved imaging technique and an experimental model where axonal transport could be easily followed by light microscopy. Previous studies had successfully used Nomarski differential interference contrast (DIC) microscopy to follow the axonal transport of mitochondria due to their rod-like large size and characteristic saltatory bidirectional movement[80,81]. Additionally, multivesicular bodies and lysosomes were identified using electron microscopy as organelles undergoing retrograde transport in a murine model[82]. Monitoring the live transport of smaller, spherical vesicles was more challenging, given the limited resolution and contrast of dark field and DIC microscopy. Video-enhanced contrast DIC (AVEC-DIC) microscopy takes advantage of adjustable black level and contrast range in video cameras to remarkably enhance resolution and recording speed, detecting particles as small as 25-50 nm[83,84]. AVEC-DIC was applied to preparations of isolated giant axons from the squid *Loligo pealeii*, which consist of a cylinder of axoplasm exceeding 500 µm in diameter, and where axonal transport can continue for up to 4 h, allowing for its visualisation in real time[85]. This setup permitted the observation of vesicles ranging in size from 30-50 nm being transported bidirectionally along the axon, with speeds corresponding to those reported earlier for fast axonal transport[86,87]. Importantly, this approach eventually led to the discovery of the motor protein kinesin[88]. This ATP-dependent motor was independently discovered in soluble fractions derived from chick brain[89]; however in both cases the crucial biochemical approach was the same: co-purification of kinesin with microtubules after treatment with the non-hydrolysable ATP analogue AMP-PNP.

In vitro motility assays using microtubules on glass coverslips showed that purified kinesin moves towards the plus ends of microtubules, which correspond to anterograde transport in axons. However, crude squid axoplasmic supernatant generates bidirectional movement, indicating the presence of a retrograde motor[90]. The purification and characterisation of the retrograde motor, cytoplasmic dynein, was done using a similar strategy, albeit in a different model. Since AMP-PNP does not promote dynein association to microtubules as efficiently as for kinesin, stabilised microtubules and their associated proteins were purified from calf brain in the absence of ATP, and then dynein was released by addition of ATP[91]. Further biochemical characterisation and *in vitro* motility assays revealed that cytoplasmic dynein is, in fact, a molecular motor which moves towards the microtubule minus end, similar to ciliary and flagellar dynein[92,93]. Dynein was independently discovered in *Caenorhabditis elegans*, a model chosen because it lacks axonemal dynein, thereby eliminating its interference in the purification of cytoplasmic dynein[94]. The role of kinesin and dynein as anterograde and retrograde motors, respectively, was confirmed *in vivo* using immunocytochemistry and electron microscopy

analyses of ligated peripheral nerves. Kinesin was found to accumulate proximal to the ligature, whilst dynein was present at both sites. In addition, both motors bound to membranous organelles[95,96]. The discovery of kinesin and dynein, and their characterisation as the molecular motors responsible for bidirectional axonal transport, closed an experimental cycle of more than 40 years that began and ended with nerve ligation experiments, and ushered in an era where motors themselves became tools to perform studies with an unprecedented level of detail.

How is axonal transport coordinated in response to internal and external cues?

Concomitant with the discovery of motor proteins and their cargoes, the last 30 years have witnessed an explosion in the availability of tools and high-resolution approaches, which has allowed a steady gain in our understanding of axonal transport. These technical advances have contributed not only to uncover *what* is transported, but also to *how* axonal transport of these cargoes is coordinated and correlated with their biological function in the neuron. Experimental strategies have focussed on the ability to label and track specific cargoes in real time as well as the manipulation of cargoes and motors to synthetically alter their positioning and motility in the axon. Additionally, *in vitro* single-molecule studies using fluorescence imaging or optical trapping have made it possible to investigate the mechanical properties of motor proteins as well as the coordination of multi-motor complexes when bound to the same cargo[97].

In an optical trap, beads coated with molecular motors are suspended in a laser beam. By changing the focus of the laser, particles can be moved with nanometer-level precision, and when combined with a surface or a second trapped particle, a force can be applied in a way reminiscent of an attached string[98,99]. These methods have made it possible to measure the force and displacement of motors at the single-molecule level; they have also led to the description of trafficking as either coordinated switching between motors (which never generate force against each other), or a tug-of-war model where dynein and kinesin simultaneously exert antagonistic forces[100]. Experiments carried out in *Dictyostelium discoideum* using endocytic vesicles suggest that 1-2 kinesins are balanced by 4-8 dynein molecules, and that dynein reduces kinesin stall force, in agreement with the tug-of-war model[101,102]. On the other hand, Kaplan and colleagues, using gold nanorods conjugated to WGA and polarisation microscopy, have demonstrated that rotation of axonal endosomes on microtubules is a consequence of shifting between kinesin- and dynein-driven motility[103]. Despite the wealth of knowledge that these techniques have yielded about the coordination of motor proteins *in vitro*[97], the applicability and translation of each of the above models to *in vivo* scenarios of bidirectional axonal trafficking remain to be fully explored.

I. Tools to label and track specific cargoes

Organelle-specific fluorescent tools

The rapid discovery of motor proteins, their adaptors and cargoes has opened new avenues to specifically label and track molecules undergoing fast axonal transport. However, it was the development of genetically-encoded fluorescent probes that proved pivotal for following a diverse range of proteins and organelles, in cultured neurons as well as in living organisms. Despite its initial discovery in 1962 by Osamu Shimomura[104], the full potential of green fluorescent protein (GFP) from *Aquorea victoria* was not uncovered until the 1990s when Tsien and Chalfie integrated it into genetically-encoded fusion proteins, eventually leading to the development of transgenic mice with GFP-tagged cellular components[105,106]. With GFP, researchers had the ability to examine the dynamics of motile organelles as never before. Following the independent discovery of DsRed from the anthozoan *Discosoma discoideum* and the

subsequent re-engineering of both GFP and DsRed, the palette of fluorescent proteins has continuously expanded and now spans the ultraviolet to the far-red regions of the visible spectrum[107], in addition to specialised monomeric photoconvertible probes which switch their optical features upon illumination[108] (**Figure 1**, orange box). This has permitted live tracking of endogenous cargoes as they move through the axon as well as the tracking of multiple cargoes in individual axons.

Given the diversity of cargoes that are transported in axons, there have been a multitude of studies that have utilised fluorescent proteins to visualise their active movement, both *in vitro* and *in vivo*. For example, kinesin-1 was shown to be necessary for the delivery of cytoplasmic dynein-GFP at hippocampal synapses[109], whilst dynactin, labelled *via* its subunit p150 with GFP, was shown to be essential for initiation of dynein-mediated retrograde transport of lysosomes[110]. Genetic alterations of kinesin-1, cytoplasmic dynein and dynactin, in combination with high-resolution imaging, revealed a vital role of dynein intermediate chain in both the anterograde and retrograde axonal transport of amyloid precursor protein (APP)-containing vesicles in *Drosophila*[111]. Vesicular axonal transport has also been studied in a *Drosophila* model of neurodegenerative disease by using a YFP-tagged human APP[112]; subsequent manipulation of this system has provided evidence of novel interactors of APP-containing vesicles with the axonal transport machinery[113]. Synaptic vesicle proteins, such as synaptobrevin and synaptogyrin, fused with GFP have enabled the live visualisation of the dynamics of synaptic vesicles in presynaptic terminals of *Caenorhabditis elegans* neurons[114] and other organisms. Murthy and colleagues have exploited transgenic nematodes expressing synaptobrevin-GFP (SNB-1::GFP) to study retrograde transport in mechanosensory neurons. The GFP tag fused to the intraluminal domain of SNB-1, was exposed to the synaptic cleft upon exocytosis and was detected by a fluorescently labelled anti-GFP antibody that was injected into the body cavity. This approach enabled the study of the retrogradely trafficking of SNB-1 vesicles[11].

Mitochondrial dynamics have been investigated in live neurons by fusing fluorescent proteins with a mitochondrial targeting sequence derived from the N-terminus of the human cytochrome oxidase c subunit VIII precursor (termed 'mito'). Expression of mito-DsRed2 was used to demonstrate that regenerating DRG neurons with high energy demands show an increase in mitochondrial traffic in the axon[115]. Fluorescent probes have also been used *in vivo* to visualise and characterise mitochondrial dynamics in the axon[116]. This seminal study, using mito-CFP and mito-YFP, pioneered analyses such as mitochondrial content and bidirectional dynamics in mouse peripheral nerves following injury and regeneration. Similar reporters (e.g. mito-eGFP, mito-DsRed and mito-mTurquoise2) enabled the interrogation of mitochondrial biology in isolated neurons[117,118], as well as *in vivo* in different animal models, such as *Drosophila,* zebrafish and mouse[116,119,120]. By using genetically-encoded photoactivable GFP and TagRFP, mitochondria have been recently tracked with nanometer precision and millisecond temporal resolution in a feedback-based, three-dimensional single particle tracking method in zebrafish larvae[121]. Indeed, these tools have facilitated the rapid expansion of *in vivo* mitochondrial transport studies, especially in the context of disease[122–125], injury[126,127] and aging[128,129].

In contrast to the approach used for mitochondria, axonal endosomes are traditionally visualised by tagging specific members of the Rab family of monomeric GTPases that are associated with their membranes on the cytosolic side[130]. Whereas fluorescent Rab5 has been widely used to study the trafficking of early endosomes, Rab11 has been used to identify recycling endosomes

that move predominantly in the anterograde direction. On the other hand, Rab7, which regulates the maturation of early endosomes to late endosomes and lysosomes, is predominantly used as a reporter of late endosomes[130]. Due to the specificity of their location, these GTPases afford greater accuracy in endosome labelling, unlike previously used molecules such as WGA. Using eGFP fused to Rab5 and Rab7, Deinhardt and colleagues demonstrated the requirement of Rab5 for the initiation of retrograde transport of signalling endosomes in murine motor neurons, while Rab7 was necessary for maintaining their long-range, dynein-dependent transport[73]. As more Rabs are being seen to be transported in the axon, they have the potential of being used to characterise other specialised organelles[131]. Since compartments along the endocytic pathway also have distinct phosphoinositide compositions, genetically-encoded probes binding specifically to PI(3)P or PI(4,5)P can be used to distinguish different endosomal populations[132]. Retrograde axonal transport of locally synthesized phosphoinositides had previously been observed by measuring incorporation of radioactive [^{3}H]-inositol injected into the sciatic nerve[133]; however, new methods allowing the identification of discreate phosphoinositide-containing membrane domains[134] have the potential to refine our understanding of endosomal sorting and trafficking along the axon.

Autophagosomes are tagged using Atg proteins involved in their formation or resident on their limiting membranes, such as LC3. Interestingly, it has been found that LC3-GFP-containing autophagosomes are formed at the distal axon and mature as they undergo retrograde transport towards the cell body in cultured neurons[135]. Importantly, their aberrant accumulation has been linked to neurodegenerative diseases[136]. Despite the generation of an LC3-GFP mouse more than 15 years ago[137], somewhat curiously, the focus of axonal transport studies has only been on isolated primary neurons. The study of axonal transport of lysosomes has typically relied on LAMP-1 or -2 fusion proteins, although acid hydrolases such as cathepsins B and D and subunits of the vacuolar H^{+}-ATPase might be better proxies for following the dynamics of functional lysosomes[138–140]. Surprisingly, the endoplasmic reticulum (ER) has received little attention with regard to its axonal transport. Earlier studies, using a variety of ER resident proteins, demonstrated that ER movement was bidirectional and tenfold slower than synaptic vesicles in cultured chick DRG neurons[141]. Similar to autophagosomes, peroxisomes have been labelled using proteins involved in their biogenesis, such as PEX3 and PEX26[142]. Alternately, the peroxisome-targeting peptide Ser-Lys-Leu has been fused to the C-terminus of fluorescent proteins to target them to the peroxisomal lumen[143].

Whereas fluorescent fusion proteins have revolutionised the way researchers investigate axonal transport, the ease of using membrane-permeant dyes to label specific axonal compartments has fuelled the discovery of novel, improved dyes (**Figure 1**, green box). Mitotracker dyes are one such option with excitation and emission wavelengths spanning the green to infra-red regions. Derived from rosamine dyes, Mitotrackers accumulate in mitochondria based on their membrane potential. However, they contain a chloromethyl moiety, making them mildly thiol-reactive and resulting in their conjugation to several mitochondrial proteins. Neurons labelled with Mitotracker can be fixed with aldehyde-containing fixatives but cannot be used for membrane potential measurements in real time[144]. Despite their limitations, these probes are widely used for monitoring the axonal transport of mitochondria in health and disease[145,146]. Rhodamine-based dyes also accumulate in mitochondria as a function of their membrane potential, but, unlike Mitotracker dyes, cannot be fixed. Because these cationic dyes do not significantly alter mitochondrial function, they have extensively been used in cultured neurons[146,147] and model

organisms[146,148], with tetramethyl rhodamine methyl ester being superior to tetramethyl rhodamine ethyl ester and rhodamine 123[149]. In addition to studying mitochondrial transport in the axon, rhodamine-based dyes have been used for assessing synaptic mitochondrial content[150] as well as mitostasis[148]. Other mitochondrial probes such as JC-1 have also been used in axonal transport studies[151], but have proved to be less reliable and more toxic[152]. Another group of commercial probes which stain axonal compartments are LysoTrackers. Spanning the visible spectrum, these dyes consist of a fluorophore conjugated to a weak base that is only partially protonated at neutral pH. As a result, these dyes preferentially accumulate in acidic compartments at nanomolar concentrations. For example, LysoTracker has been used to study the impaired transport and accumulation of lysosomes in cortical neurons derived from a mouse model of Alzheimer's disease[153]. However, it should be noted that LysoTracker dyes accumulate in organelles based on their acidity, hence labelled compartments might not specifically be lysosomes. There is scant evidence of other axonal compartments being labelled with free dyes. Farias and colleagues have recently applied ER-Tracker Red to label the ER and follow its transport in primary rat hippocampal neurons[154]. This cell-permeant dye consists of Bodipy-labelled glibenclamide, which binds to sulphonylurea receptors on the ER. Counter-effects of the glibenclamide moiety on ER function, if any, are currently unknown.

Incubation of neurons with exogenous fluorescent probes has been a powerful approach to study the mechanisms and dynamics of retrograde transport. After the elucidation of their mechanism of action, fluorescent derivatives of cholera and tetanus toxins have been validated as reliable markers of retrograde signalling endosomes[155]. The co-application of neurotrophic factors with HcT-GFP was used to determine modulators of endocytosis and axonal transport of signalling endosomes. Indeed, the neurotrophic factors BDNF, NT-3, NT-4, GDNF and CNTF all enhance the accumulation of HcT in ligated sciatic nerves, demonstrating that these factors are enhancers of endocytosis and/or axonal transport[156]. In particular, fluorescent HcT has proven to be a valuable tool for mapping the molecular composition and maturation of signalling endosomes in neurons[73,157]. Using *in vivo* imaging of fluorescent HcT injected in muscles, Bilsland and colleagues demonstrated axonal transport deficits in a mouse model of amyotrophic lateral sclerosis (ALS)[158]. Further optimisation of this approach[159] has allowed the investigation of pathomechanisms in several murine models of neurological disease, including spinal and bulbar muscular atrophy[160] and ALS[161,162]. On the other hand, Meunier and colleagues have shown that TrkB signalling is a primary determinant of retrograde signalling endosome flux in hippocampal neurons using CTB[77]. Despite widespread use in cultured cells, the *in vivo* use of CTB has been limited[123].

Cell surface molecules, such as neurotrophin receptors and their ligands, have been widely exploited to study axonal transport dynamics. However, specific strategies had to be developed to follow their physiological trafficking. Assays using radiolabelled neurotrophins have poor spatiotemporal resolution and could not be used for live imaging[163], whilst antibodies recognising them would often influence their biological activity[63]. In addition, genetically-expressed fluorescent neurotrophins, although widely used, are targeted for secretion and therefore their use to study endocytosis and retrograde transport of target tissue-derived neurotrophins is not straightforward. Recently, neurotrophic factors have been conjugated to more advanced fluorescent probes, generating sophisticated tools, such as quantum dot-labelled BDNF[164] and AlexaFluor488-NGF[165], permitting single particle tracking in live cells[166] and giving insights into the identity of organelles carrying neurotrophic signals. Concomitantly, the characterisation of

several highly specific commercial antibodies has enabled the tracking of membrane-bound receptors and other plasma membrane molecules. A fluorescently labelled antibody against the p75NTR neurotrophic receptor demonstrated that after addition of NGF, internalization rates and kinetics of p75NTR-containing signalling endosomes are slower than those carrying Trk receptors[167]. Synaptic vesicles have also been labelled using an anti-cysteine string protein (CSP) antibody and the accumulation of fluorescent CSP antibody was used as a proxy for axonal transport in a *Drosophila* tau model[168].

Fluorescent probes for functional studies

Altogether, the fluorescent probes described above have enabled the spatiotemporal tracking of axonal cargoes. However, each organelle undergoing axonal transport is characterized by a chemical microenvironment, which is intimately linked to its neuronal function. Thus, while lysosomes require acidic pH to degrade macromolecules, the mitochondrial matrix has an alkaline pH due to the coupling of ATP production and outward proton pumping. Wild type GFP is inherently sensitive to changes in pH due to protonation of its chromophore (pK_a ~6.1)[169] and has been used as a genetically-encoded pH sensor[170]. Rational mutagenesis of GFP has resulted in superior pH indicators such as ratiometric and ecliptic pHluorins; the former shows two reversible, pH-sensitive excitation peaks, which allows the ratiometric detection of the luminal pH of the targeted organelle, such as synaptic vesicles[171] and signalling endosomes[172]. Single wavelength changes exhibited by many fluorescent proteins are exploited by creating tandem pH indicators using a second pH-insensitive fluorophore; these include super-ecliptic pHluorin-mKate2, GFP-mCherry or GFP-RFP[170,173,174]. Recently, Shen and colleagues have described an RFP-based pH indicator, called pHuji, which shows a 20-fold change in fluorescence intensity between pH 5.5 and 7.5[175].

A limitation of these probes is that lysosomal hydrolases, which are an indicator of functional lysosomes[176], are thought to be active at pH ~5.0 or below, which lies outside the linear detection range of these pH sensors. The only probe which retains its sensitivity at such low pH values is the fluorescence resonance energy transfer (FRET) pair consisting of pHlameleon 5 and YFP Venus[177]. Whereas the development of genetically-encoded pH sensors has seen great progress in the last 20 years, their application to study axonal transport has been limited. Recently, the mito-Keima mouse, which expresses a pH-sensitive, dual-excitation ratiometric fluorescent protein, was developed to study mitophagy[178]. In this model, there is a switch of excitation wavelengths, from 440 nm at physiological mitochondrial pH (~8.0) to 586 nm as mitophagy induces an acidic shift (~4.5)[179]. This model has allowed the visualisation of mitochondrial turnover[180] and mitophagy in neurodegenerative models[181,182]. However, while resistant to lysosomal hydrolases, the Keima moiety is incompatible with fixation as well as *in vivo* multi-colour imaging. To circumvent these limitations, the Ganley group has developed the mito-Quality Control (mito-QC) mouse which expresses a tandem mCherry-GFP protein fused to the signal sequence of FIS1, an outer mitochondrial membrane protein. This genetically-encoded, ratiometric pH sensor was used to report on mitochondrial architecture and turnover in several murine tissues, including specific subsets of cerebellar neurons[183].

Production of small molecules as by-products can also be used to shed light on organelle function and its axonal transport. Several ATP indicators have been used to assess mitochondrial activity, including ATeam, BTeam, ARP-1 and RSL+[184], but to date, none of them has been used for examining the spatial correlation between axonal transport and mitochondrial function. Probes

sensitive to the generation of reactive oxygen species (ROS) provide no information about local changes within the organelle or have different degrees of pH sensitivity[185]. MitoSOX has been used to correlate ROS and axonal transport in the context of Friedreich ataxia[186] and β-amyloid toxicity[187]. However, more sensitive and specific probes are needed to resolve changes in ROS identifying certain mitochondria pools. Given the importance of oxidative stress, GFP-based sensors, such as roGFPs, have been developed to detect changes in cellular redox potential. RoGFPs contain engineered cysteines that, based on the environmental redox potential, switch between dithiol and disulphide states, leading to changes in fluorescence[188,189]. When fused to the glutaredoxin Grx1, roGFP2 is able to report on redox signals associated with gluthathione[190]. This fusion protein, when targeted to neuronal mitochondria, was used to investigate changes in mitochondrial dynamics as a function of redox potential in mouse models of ALS and spinal cord injury[191].

Calcium, which is often used to monitor neuronal activity, is known to influence fast axonal transport of multiple cargoes[192]. This has been of particular interest in mitochondria, given the high energy consumption associated with neuronal activity and the ability of mitochondria to control the concentration of cytosolic calcium. The functional coupling of intracellular calcium levels and mitochondrial transport requires the mitochondrial calcium sensor Miro1, which acts as a kinesin adaptor[193]. Currently, genetically-encoded calcium indicators (GECIs) are mainly used to visualize neuronal activity[194]. GECIs typically comprise a calcium-sensitive domain, usually calmodulin (CaM), fused to one or two fluorescent proteins. The principle behind fluorescent GECIs relies on the conformational change induced in the CaM domain in the presence of high calcium concentrations. In single wavelength GECIs, such as GCaMPs, the calcium-binding moiety is embedded within a circularly permutated fluorescent protein; binding of calcium leads to the formation of a fluorescent chromophore. In contrast, dual wavelength GECIs, such as Cameleons, consist of a FRET pair present on either side of CaM, which undergo energy transfer upon calcium binding[195]. In a corticostriatal neuronal network, the axonal motility of mitochondria and dense core vesicles was found to depend on the developmental status of the network, measured by GCaMP6 signal[196]. Additionally, the GECI Case12 was used to demonstrate the correlation between mitochondrial motility and calcium concentration in the mitochondrial matrix in mouse hippocampal neurons[197].

Conventional approaches for the manipulation of intracellular calcium include the application of calcium chelators, changing concentrations of extracellular calcium and treatment with calcium channel agonists or inhibitors. These treatments, however, have little to no spatial specificity. More recently, some groups have developed tools to control calcium mobilization called genetically-encoded calcium actuators (GECAs), which allow the control of intracellular calcium with subcellular precision[198]. A GECA which was successfully tested *in vivo* in *C. elegans* neurons is based on the photosensitive LOV2 protein fused to a CaM-M13 fusion protein[199]. CaM-M13 forms a complex that can bind calcium at physiological resting concentrations (K_d ~16 nM), thus acting as a chelator. Upon blue light exposure, the CaM-M13 complex gets disrupted, causing the local release of calcium. More recently, a tool capable of controlling calcium release from the ER was developed[200]. This tool is formed by the Channelrhodopsin-green receiver, which possesses calcium permeability, fused to an ER retention signal sequence. Blue light activates the channel and allows the local release of calcium. Given the almost ubiquitous localisation of the ER in neurons, this tool is extremely promising in permitting the subcellular control of calcium transients.

Techniques for enhancing transport resolution in vitro and in vivo

The evolution of a large axonal transport toolkit has been matched by an equally wide growth in microscopic techniques to view axonal cargoes in real time (**Figure 1**, blue box). While the first evidence of axonal transport was obtained using time lapse DIC microscopy[83,84], the field has greatly benefitted from major advances in widefield fluorescent microscopy. However, imaging of axonal trafficking *in vivo* poses additional challenges. For instance, tissue samples scatter more light above and below the focal plane due to their thickness, reducing the signal-to-noise ratio (SNR). Confocal microscopy employs a pinhole to limit the field of illumination and visualisation, scanning the specimen with a discrete light source, and removing out-of-focus light from planes above and below the optical plane. The increased axial resolution and SNR of confocal microscopy has allowed the *in vivo* tracking of axonally transported fluorescent proteins, such as rhodamine-NGF in superior cervical ganglion neurons in culture[201], or the monomeric kinesin motor UNC-104-GFP in *C. elegans* neurons[202]. Despite its relative low scanning speed, confocal microscopy is currently used for imaging axonal transport *in vivo*[10]. Spinning disk microscopy, on the other hand, offers increased acquisition speed while reducing phototoxicity and photobleaching, making this technique very useful to image membrane trafficking[203]. This method has been used for imaging axonal transport of secretory vesicles and mitochondria in microfluidic chambers[196], as well as in the adult *Drosophila* wing[119]. Both confocal and spinning disk microscopy have also aided the study of molecular interactions through FRET and fluorescence lifetime imaging, such as signalling proteins associated to endosomes[204].

Even though the point-scanning techniques mentioned above avoid collecting out-of-focus fluorescence signal, the tissue is still exposed to the risk of photodamage. Several microscopy techniques have now been developed to enable imaging of deep tissues with minimal photo-toxicity and bleaching, such as two-photon and light sheet microscopy. While two-photon microscopy has been used for monitoring cargo dynamics in rat hippocampal slices[205], zebrafish neurons[126] and mouse brain and spinal cord[124,206], light sheet microscopy has enabled the high speed, three-dimensional imaging of Rab5-positive endosomes in retinal pigment cells moving as fast as 10 μm/s[207].

Besides reduced phototoxicity, *in vivo* axonal transport imaging needs to provide high spatial resolution to investigate the relative position of different transported molecules. Super-resolution microscopy, such as single molecule localisation microscopy (SMLM) and stimulated emission depletion (STED) have been used in the nervous system *in vivo*[208,209]; however, the high laser intensities of the former and the long acquisition times of the latter make it difficult to use them for axonal transport. Correlative light and electron microscopy (CLEM) takes advantage of merging light and electron microscopy analyses of the same sample, to describe dynamic and ultrastructural details[52]. Despite being technically challenging, CLEM has been used for observing neurofilaments moving as polymers during slow axonal transport[210]. Another super-resolution approach is structured illumination microscopy (SIM), which employs a rotating lattice of light to increase resolution. Multi-colour SIM is well suited for high-speed *in vivo* imaging, and has been used for tracking new KIF1Bβ cargoes in cultured neurons[211]. Most recently, time-lapse SIM was used to assess the influence of actin ring dynamics on axonal transport of endosomes and lysosomes[212]. Interestingly, lattice illumination patterns can also be applied to light sheet microscopy, allowing sub-resolution, three-dimensional imaging of neurons from whole animals[213,214]. Further developments pertaining to STED and SMLM[215,216], analytical approaches

applicable to a wide range of light microscopy, such as super-resolution radial fluctuation (SRRF)[217] and the combination of these with correlative microscopy[218] will enable the imaging of cargoes and motors at an unprecedented spatiotemporal resolution *in vitro* and *in vivo*.

Organelles undergoing anterograde and retrograde transport along the axon are not uniformly distributed, but mature and evolve in time and space. Lysosomes and autophagosomes are thought to become progressively acidified in their journey from distal axons to the soma[219]; mitochondria undergo fission and fusion, as their membrane potential and production of ATP and ROS varies[220,221]; early endosomes mature into late or recycling endosomes, some of which have crucial signalling properties[222], to name a few examples. With the rich diversity of probes, tools and techniques available to study axonal trafficking, it is now possible to study multiple cargoes at the same time and understand their function as well as transport dynamics. Multiplexing has permitted a more detailed characterization of organelles using a combination of reporters, as was done for signalling endosomes using HcT along with BDNF and its receptors TrkB and p75^{NTR}[73]. This approach also facilitates the simultaneous observation of different populations of organelles, as demonstrated by Che and colleagues who utilized TrkA-mCherry and Mito-YFP or LAMP1-mCherry and Mito-YFP to infer that cargoes undergoing fast axonal transport move slower in the vicinity of stationery organelles[223]. Using dual-colour imaging, proteasomal subunits have been found to undergo coordinated transport with pre-synaptic vesicles, lysosomes and mitochondria in hippocampal neurons[224]. Interestingly, RNA granules have also been observed to 'hitchhike' on lysosomes, using the phosphoinositide-binding protein annexin-11 as a linker[225].

Monitoring changes in an organelle undergoing axonal transport can be achieved by fusing a functional reporter with a transport probe. A fusion protein linking ratiometric pHluorin with HcT was used by Bohnert and colleagues to demonstrate that HcT-carrying retrograde vesicles escape the acidification normally associated with endosomal maturation[172]. Axonal retrograde carriers display a neutral pH that is maintained during their transport towards the cell body, and suggests how tetanus toxin evades identification and degradation in host neurons[172]. Similarly, the autophagosome marker LC3 has been used in tandem with GFP and RFP to constitute a dual fluorescent ratiometric sensor. Relying on the pH sensitivity of GFP and insensitivity of RFP, this probe was used to study the maturation of autophagosomes to autophagolysosomes[170]; however, given the pH range of wild type GFP[169], whether this organelle can be defined as a true autophagolysosome is currently controversial.

Multiplexing capabilities have continued expanding, both by new methods of analysis[226] as well as techniques such as nucleotide-based transient labelling. These techniques, notably among them DNA points accumulation for imaging in nanoscale topography (DNA-PAINT), rely on the programmability and stability of DNA to accurately locate multiple cellular proteins using super-resolution microscopy[227]. In the future, these methods are expected to provide unprecedented specificity for classifying the gamut of organelles transported along the axon.

Given their unique morphologies and cell-cell interactions, neurons are subject to very diverse environmental conditions *in vivo*. As such, it is not surprising that the dynamics of axonal transport observed *in vitro* present significant differences with those observed *in vivo*[118,122,159]. While axonal transport studies have predominantly been carried out in cultured neurons, a number of experimental setups have been devised to either emulate *in vivo* conditions *in vitro*, or to enable *in vivo* imaging of axonal transport. Compartmentalised chambers, such as microfluidic

devices, achieve fluidic isolation of the cell body from the axon terminal, creating experimentally-controlled microenvironments. This enables the localised application of probes as well the study of how extracellular cues modulate axonal transport in different neuronal types[228]. Inspired by Campenot chambers and fabricated from the silicone resin poly(dimethylsiloxane), microfluidic devices are optically transparent and have been used to image single molecules of NGF labelled with quantum dots in DRG neurons[229]. Medioni and colleagues have exploited microfluidic chambers to grow *Drosophila* brain explants and study the dynamics of ribonucleoprotein complexes in mushroom body neurons[230]. Using larval stages of *C. elegans* and *Drosophila*, they have been used to perform live imaging of various cargoes (Rab3, OSM-6 and ODR-10) in a whole-mount animal throughout its development[231]. This innovative experimental approach also helped to offset the potential impact of anaesthetics on axonal transport. *In vivo* imaging using non-invasive methods has been facilitated by the use of genetically tractable and optically transparent animals such as *C. elegans* and zebrafish embryos and larvae, or tissues such as the *Drosophila* wing[119,231,232]. For example, time-lapse microscopy has been used to capture endosomal dynamics (Rab5c, Rab7, Rab11a) as well as axonal outgrowth and branching in zebrafish central and peripheral axons[233]. In contrast, while several human neurodegenerative diseases have been modelled in mice, *in vivo* study of axonal trafficking in the murine nervous system relies on injections of relevant probes in target tissue(s) and invasive surgical interventions[122,162,234] (for a more in-depth review on *in vivo* imaging of axonal transport, please refer to ref. 10).

Advances made in the field of fast axonal transport using *in vitro* and *in vivo* experimental approaches have translational potential, bridging the gap between pre-clinical and clinical studies. Magnetic resonance imaging (MRI), developed with a manganese-based approach, relies on the ability of manganese ions to act as calcium analogues. These manganese ions are internalised into neurons through voltage-gated calcium channels and undergo axonal transport along microtubules, a process that can be visualised using MRI[235,236]. In this approach, called manganese-enhanced MRI (MEMRI), manganese was targeted to the olfactory system *via* intranasal delivery. MEMRI was used for trans-synaptic tracing[237] as well as in mouse models of Alzheimer's disease[238,239] and schizophrenia[240]. Alternatively, manganese can be delivered by intra-ocular or stereotaxic hippocampal injections and imaged upto 24 h after delivery[241]. Using MEMRI and mice lacking the kinesin-1 light chain, the transported manganese signal in different cortical regions distal from the injection site was used to tease apart the functional role of this kinesin-1 subunit in motor activity[242,243]. In addition, positron emission tomography (PET)-based axonal transport probes targeting Trk receptors have recently been developed for human use, allowing the imaging of their accumulation in cortical brain regions[244,245].

Genetic tools

The development of this innovative molecular toolbox has been mirrored by an expanding genetic toolbox enabling the visualisation of tagged proteins and their mutant alleles in specific neuronal subpopulations. Indeed, genetic tools, including Cre-LoxP recombination, viruses (such as adeno-associated virus) and CRISPR-Cas9 technology, have allowed spatial and temporal control of gene expression *in vivo*. Pioneered by Martin Evans, Oliver Smithies and Mario Capecchi, Cre-LoxP has been used to delete or knock in target genes containing LoxP sequences in multiple model organisms[246]. Spatial control of transgene expression is achieved using cell type-specific promoters, while inducible Cre-systems permit temporal and spatial control of transgene expression. However, introduction of Cre-LoxP sites into genomes may cause unexpected phenotypes[247], while clearance of tamoxifen can take several days, depending on the genetic

background[248]. Due to its potential pitfalls, Cre-LoxP recombination is slowly being replaced by zinc finger nucleases, transcription activator-like effector nucleases and CRISPR-Cas9-mediated genetic manipulation[249]. CRISPR-Cas9 was discovered in bacteria as a defence mechanism against viruses[250] and has revolutionised the production of transgenic and mutant organisms by enabling precise, targeted modifications of the genome[251]. The specificity of Cas9 targeting and the possibility of multiplexing guide RNAs yield very high recombination efficiencies[251]. Using these genetic tools, investigators are now able to target a particular subset of neurons, which in combination with other axonal transport approaches (e.g. HcT, LysoTracker, crossbreeding with reporter mice, such as those expressing mito-YFP) allow axonal transport studies of an organelle in a defined neuronal subpopulation *in situ*. Using these combinatorial approaches, it was recently shown that *in vivo,* signalling endosomes have different axonal transport dynamics in DRG sensory neurons compared with cholinergic motor neurons[162]. As neurons have their own specific functions, metabolic requirements and anatomical properties[252], these results suggest that the axonal transport dynamics of certain organelles may have different speed profiles in different neurons, and their transport dynamics might be differentially altered in disease.

II. Tools to manipulate positioning and axonal transport of cargoes

In addition to probes enabling the identification and tracking of specific axonal organelles, a variety of tools have been developed to manipulate axonal trafficking and study its mechanisms. Classical strategies have involved the use of drugs that either destabilize microtubules, such as colchicine[253], vincristine[254], maytansine[255] and nocodazole[256], or drugs that inhibit transport from the ER to the Golgi complex, such as brefeldin A[257]. However, since these drugs have major effects on the integrity of the cytoskeleton, they disrupt other cellular processes, thus hindering a detailed dissection of the molecular mechanisms of axonal transport. Therefore, kinesin and dynein inhibitors have been developed in an attempt to circumvent these issues. Whereas this has been possible for cytoplasmic dynein[258], which exists as a single isoform, this task has been more challenging for kinesin, which is present in several different isoforms with cell-specific expression patterns. Nonetheless, drug discovery initiatives have yielded promising kinesin inhibitors, which impair cell division by blocking spindle formation[259].

Inducible dimerization systems offer the possibility to experimentally control protein-protein interactions by driving the association between two proteins in the presence of a physical or chemical dimerizer. Inducible dimerization systems have been used to link specific organelles to molecular motors with great temporal and spatial control. A commonly used system is based on the ability of FK506 binding protein 12 (FKB12) to irreversibly bind rapamycin with nanomolar affinity[260]. The FKB12-rapamycin complex can bind a component of the mTOR pathway named FRAP (FKB12-rapamycin associated protein)[261,262], which has been renamed FBP (FKB12 binding protein; also referred to as FRB). This property allows the use of the membrane-permeant small molecule rapamycin (or its more recent analogue rapalog) to control the dimerization of target proteins fused to FKB12 and FBP proteins[263,264]. The Hoogenraad and Kapitein groups have pioneered the FKB12-rapamycin-FRB system to investigate axonal transport[142,265–268]. This approach has allowed the study of the mechanism by which the protein Bicaudal D2 (BICD2) recruits cytoplasmic dynein and acts as motor adaptor[265,269]. This was possible by fusing BICD2 to FBP and a mitochondrially targeted GFP to FKBP; the addition of rapalog forced the binding of BICD2 to mitochondria, which was sufficient to drive the transport of these organelles to the cell body[265]. More importantly, fusion of target organelles to BICD2, and thus to dynein, was found to promote bidirectional transport on microtubules of mixed polarity present in dendrites[266,267]. The

ability to control specific kinesins led to the observation that the microtubule associated protein MAP2 inhibits the binding of cargoes to a slow motor (kinesin-1) at the proximal axon in sensory neurons, whilst promoting their binding to a fast motor (kinesin-3), thus acting as a filtering system. The absence of MAP2 in distal axons allows for cargoes to bind kinesin-1 and be delivered to their target location[268]. This approach has also been used in an interesting study that screened different kinesin tails for their specific cargo binding. By fusing the constitutively active motor domain of KIF5C to FKBP and different kinesin tail domains to FBP, the Banker group was able to assess the change in transport of fluorescently-tagged vesicular marker proteins upon rapalog addition. This demonstrated that vesicles containing the transferrin receptor (TfR) are transported by KIF13A and KIF13B whereas low-density lipoprotein receptor-containing vesicles are transported by KIF1A and KIF13B[270].

An alternative dimerization approach uses an analogue of the plant hormone gibberellin to drive the interaction between two proteins, the gibberellin insensitive dwarf1 (GID1) and gibberellin insensitive (GAI)[271]. This system was used in combination with the FKBP-FBP system, thus enabling differential control of two motors simultaneously. Using this approach, it was shown that the actin-dependent motor myosin-V participates in vesicular sorting at the axon initial segment by stalling kinesin-1-bound vesicles destined for dendrites[272]. A similar approach takes advantage of the well described femtomolar interaction between streptavidin and biotin. This system, named RAMP (reversible association with motor proteins), is based on fusing two target proteins to either streptavidin or a streptavidin-binding peptide (SBP), enabling forced interaction between them. Unlike FKB12-rapamycin-FRB binding, this interaction can be reversed by the addition of exogenous biotin[273]. Other similar approaches are the RUSH (retention using selective hooks) and CUTE (controlled unmasking of targeting elements) systems. RUSH relies on a hook protein fused to streptavidin whilst a target protein is fused to SBP; binding of streptavidin to SBP ensures that the target protein remains indirectly docked to the hook protein and thus, localised in the donor compartment. Addition of biotin disrupts this interaction and causes a synchronous release of the target protein[274]. The CUTE system, on the other hand, consists of SBP fused to a target protein. Binding of streptavidin to SBP causes the masking of the signal sequence of the target protein, thus trapping it into a donor compartment. Protein transport to the target compartment is achieved by unmasking the signal sequence *via* addition of excess biotin[275]. Although both these methods hold promise and have been used to study transport in HeLa cells[274,275] and polarised sorting of neuronal proteins at the Golgi complex[276], their application in studying axonal transport has been limited.

The chemical-induced dimerization methods described above offer temporal control over axonal transport; however, the engineering of proteins whose dimerization can be reversibly controlled by light allows for even greater temporal *and* spatial control. In recent years, Van Bergeijk and colleagues have described TULIP (tunable light-inducible dimerization tags) which is based on a photosensitive LOV (light-oxygen-voltage sensing) domain from the protein phototropin 1 that, upon exposure to blue light (<500 nm), can bind to an engineered PDZ domain (ePDZb1)[277,278]. Dimerization is achieved within seconds and is nearly completely reversible with similar kinetics once the illumination source is removed. By fusing the LOV domain to specific organelle markers and the ePDZ domain to motor proteins, this tool was successfully used to mobilise lysosomes, mitochondria and Rab11-positive vesicles in neurons[278]. Similar tools successfully tested in neurons are Magnets[279,280], which use a modified Vivid blue light photoreceptor (VVD) from *Neurospora crassa*, and the CRY2-CIB1 system[281].

Since the reversibility of dimerization can be a potential issue for several of the experiments described above, some groups have developed photoactivable dimerizers that bind with increased stability. One such tool is based on the Halotag system, which uses a photocaged molecule of trimethoprim (cTMP) linked to a HaloTag ligand (cTMP-Htag). Upon excitation at 400 nm, cTMP is uncaged, freeing the Halotag ligand and enabling its covalent association with the Halotag protein; following this, the dihydrofolate reductase (DHFR) protein binds to the Halotag complex. Caged cTMP-Htag is stable upon illumination at 488 nm and can, thus, be coupled with commonly used axonal transport probes and GFP-based fluorescent proteins, unlike the TULIP method. Additionally, the Halotag complex can be dissociated by adding free TMP in the cell media[282]. This tool has been successfully used in primary neurons to recruit motor proteins to specific organelles[282,283]. Reversibility has also been achieved in the FKBP-FBP system by engineering a mutant FKBP that forms homodimers with micromolar affinity, thereby eliminating the need for FBP in the dimerization process. These homodimers can be disassembled by application of synthetic ligands[284,285]. Mutant FKBP has been used to induce ER accumulation of TfR and subsequent release upon addition of the ligand, creating an easily detectable wave of transport. This method demonstrated that TfR, normally targeted to dendrites, travels towards axons, but is stopped and directed back to dendrites by actin and myosin-Va[286]. Similarly, a light-inducible secretion system has been developed using the photoreceptor protein UVR8, which forms dimers that can be reversibly dissociated by exposure to ultraviolet light at 300 nm[287]. UVR8 was used to release VSV-G trapped in the ER and induce its transport to the Golgi complex in neurons, leading to the characterisation of a novel role for synaptotagmin 17 in this process[288].

A fully inducible-reversible system has recently been developed for transport experiments in neurons[289]. This is based on a small molecule called zapalog, which consists of a synthetic ligand of FKBP (SLF) conjugated to the DHFR ligand TMP with a photocleavable linker (TMP-SLF). Both TMP and SLF bind to their respective receptors with sub-micromolar affinities and lack endogenous targets. Application of zapalog to cells causes dimerization of the two proteins whereas exposure to 405 nm light cleaves the TMP-SLF adduct, disrupting the interaction between FKBP and DHFR. This reaction is completely reversible, since re-administration of zapalog can restore dimerization. Its stability to wavelengths above 488 nm makes it suitable for live imaging. Using this system, Gutnick and colleagues were able to identify a tightly anchored pool of mitochondria localised at presynaptic sites in neurons that are insensitive to kinesin binding[289].

In comparison to chemical and photo actuators, the use of physical stimuli to manipulate axonal trafficking has been relatively limited. Recently, an antibody against the neurotrophin receptor TrkB was functionalized with magnetic nanoparticles and loaded into signalling endosomes in DRG neurons[290]. The transport of TrkB-carrying organelles was then modulated by application of a magnetic field. A similar approach was applied to DRG neurons grown in microfluidic chambers and was used to show that signalling endosomes do not detach from microtubules during pauses[291].

Other tools to manipulate neurons with greater spatiotemporal resolution have also been recently described. One such approach uses artificial vesicles, such as liposomes, which are loaded into cells by microinjection[292,293]. Liposomes of different sizes (30-300 nm) were labelled with phosphatidylethanolamine-Halotag ligand conjugate and microinjected into HeLa cells

expressing a Halotag protein targeted to the outer membrane of mitochondria, thus enabling the recruitment of these artificial vesicles to mitochondria[292]. In another experiment, the insertion of a specific set of SNARE proteins into liposomes allowed the recruitment of these artificial vesicles to early endosomes[293]. These experiments demonstrated that this approach allows the controlled delivery of proteins to specific organelles to manipulate their function, transport or both. The main limitation of this system lies in the delivery of liposomes to neurons, since these cells are extremely sensitive and microinjection may alter their function. These approaches are yet to be tested in the context of axonal transport, but have the potential to unravel how different molecular interactions and functional states affect the positioning of organelles and their axonal dynamics.

Future perspectives

The burgeoning transport toolkit has enabled the dissection of axonal trafficking with remarkable precision. We envisage that application of innovative tools being developed in other fields will further assist in addressing important open questions in the field. In this regard, we have limited understanding about the physiological cues, either cytosolic or intrinsic to certain organelles, that affect axonal transport. These include neuronal activity, membrane potential, ATP, metabolites (e.g. lactate) and ion concentrations, including calcium and pH. Tools to study and modulate these parameters are currently available, such as microbial rhodopsins[294] and genetically-encoded voltage indicators[295–298] but their use in combination with the study of axonal transport has been limited. Newer genetically-encoded probes, which provide combinatorial sensing properties, have also been described[299]. DNA-based sensors, in particular, provide programmability and targetability in a variety of cellular contexts[300] and have been used to measure pH[301,302], chloride[303,304], calcium[305] as well as enzymatic activity[306]. The development of highly specific, high affinity single-domain antibodies, or nanobodies, has enabled the investigation of the molecular changes that accompany organelle trafficking and maturation. Since they are small and can be subjected to directed evolution *in vitro*, nanobodies can be used to distinguish between highly homologous transport proteins as well as different conformational states of a protein; additionally, they are often stable to changes in pH and temperature[307,308]. While nanobodies can help in the visualisation of motile proteins, further improvements of microscopic techniques, such as correlative light and electron microscopy, offer an opportunity to explore both dynamics and ultrastructure of confounding organelles, such as multivesicular bodies and tubular endosomes[222]. Additionally, induced pluripotent stem cells provide a promising platform to dissect molecular mechanisms of axonal transport in a variety of neurodegenerative contexts[309].

As more model systems and probes become available to make an accurate assessment of axonal transport *in vivo*, the field will benefit from the integration of artificial intelligence and deep learning strategies. These will be especially useful to glean patterns from big datasets in an unbiased manner[310], particularly those generated by studies employing multiplexing of several probes *in vivo*. With the variety of tools on offer, it should soon then be possible to build *systems biology* transport models predicting the precise coordination of axonal trafficking as a function of its intrinsic and extrinsic environmental factors.

Figures.

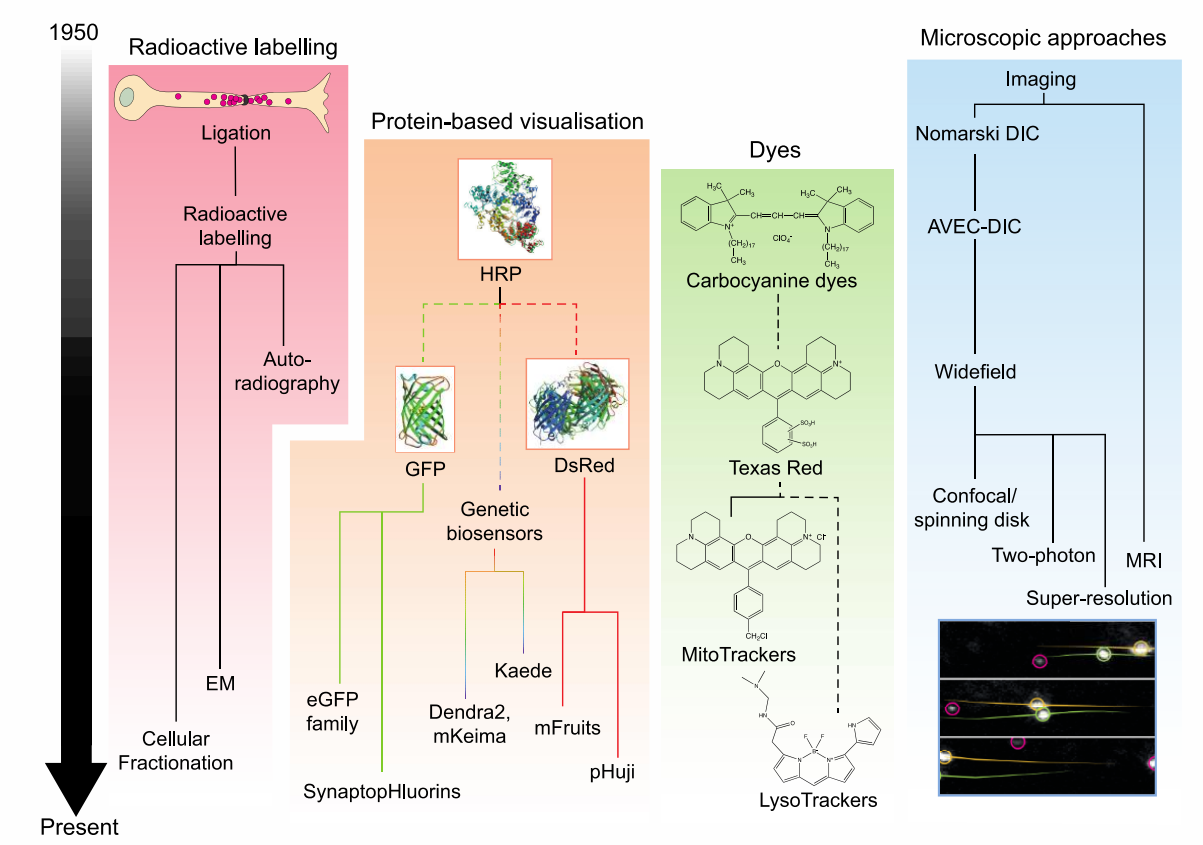

Figure 1. Schematic showing the evolution of tools and techniques to visualise axonal transport of cargoes in neurons. In early studies, labelling of newly synthesised biomolecules using radioactive precursors was the technique of choice. Radioactivity was then assessed using autoradiography coupled to electron microscopy (EM), or cellular fractionation **(red box)**. Protein-based tools, such as horseradish peroxidase (HRP; PDB ID: 1ATJ[311]), offer ease of labelling; however, after the discovery and subsequent engineering of green fluorescent protein (GFP; PDB ID: 1EMA[312]) and DsRed (PDB ID: 1G7K[313]), a great variety of fluorescent proteins with different excitation/emission wavelengths, photophysical properties and ion sensitivities became available **(orange box)**. Small molecule dyes have also undergone massive development from carbocyanine dyes to a palette of mitochondria-specific Mitotrackers and acidic organelle-specific Lysotracker dyes **(green box)**. The expansion in protein- and dye-based probes has been matched by transformative developments in microscopy-based techniques, from Nomarski differential interference contrast (DIC) and video-enhanced contrast DIC (AVEC-DIC) microscopy to widefield, confocal, two-photon, super-resolution and magnetic resonance imaging (MRI) methods. Bottom: Representative image demonstrating particle tracking of signalling endosomes labelled with the atoxic fragment of the tetanus toxin (HcT) and imaged using a confocal microscope **(blue box)**.

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