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# *In vivo* imaging of axonal transport in murine motor and sensory neurons

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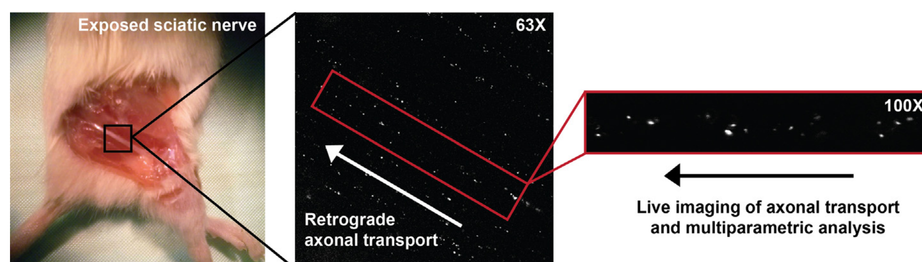
## HIGHLIGHTS

- *In vivo* imaging of axonal transport in the sciatic nerve of live anesthetised mice.
- Signalling endosomes are monitored using fluorescent probes and confocal microscopy.
- Axonal transport in motor and sensory neurons can be differentiated.
- This method can be easily adapted to study the axonal transport of other cargoes.
- Potential use of this *in vivo* imaging approach in drug screening.

## GRAPHICAL ABSTRACT

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## ABSTRACT

*Background:* Axonal transport is essential for neuronal function and survival. Defects in axonal transport have been identified as an early pathological feature in several disorders of the nervous system. The visualisation and quantitative analysis of axonal transport *in vivo* in rodent models of neurological disease is therefore crucial to improve our understanding of disease pathogenesis and for the identification of novel therapeutics.

*New method:* Here, we describe a method for the *in vivo* imaging of axonal transport of signalling endosomes in the sciatic nerve of live, anaesthetised mice.

*Results:* This method allows the multiparametric, quantitative analysis of *in vivo* axonal transport in motor and sensory neurons of adult mice in control conditions and during disease progression.

*Comparison with existing methods:* Previous *in vivo* imaging of the axonal transport of signalling endosomes has been limited to studies in nerve explant preparations or non-invasive approaches using magnetic resonance imaging; techniques that are hampered by major drawbacks such as tissue damage and low temporal and spatial resolution. This new method allows live imaging of the axonal transport of single endosomes in the sciatic nerve *in situ* and a more sensitive analysis of axonal transport kinetics than previous approaches.

*Conclusions:* The method described in this paper allows an in-depth analysis of the characteristics of axonal transport in both motor and sensory neurons *in vivo*. It enables the detailed study of alterations in axonal transport in rodent models of neurological diseases and can be used to identify novel pharmacological modifiers of axonal transport.

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## 1. Introduction

Neurons are highly polarised cells with axons that can extend over a meter away from the cell body in large mammals. This unique morphology makes neurons particularly reliant on active intracellular transport. Long distance ATP-dependent transport along the axon is predominantly mediated by two families of microtubule-associated motor proteins: kinesins and cytoplasmic dynein (Maday et al., 2014). Axonal transport plays two main roles in neurons: (1) Supply and clearance: delivery of organelles and newly synthesised proteins, lipids and RNA to the axon and distal synapses, and removal of faulty ones to the soma for degradation and/or recycling; (2) Long-distance communication between the axon tip and the soma, including the transport of neurotrophic factors and their activated receptor complexes, which are essential for survival (Chowdary et al., 2012). It is therefore not surprising that alterations in axonal transport can be deleterious to neuronal function and survival, and that such deficits are proposed to be involved in the pathogenesis of several neurological diseases, including amyotrophic lateral sclerosis (ALS) (Bilsland et al., 2010; Kieran et al., 2005), Charcot Marie Tooth type 2 (d'Ydewalle et al., 2011), hereditary spastic paraplegia (Kasher et al., 2009) and diabetic neuropathy (Tomlinson and Mayer, 1984).

Due to the well-documented relationship between mitochondrial defects and neurodegenerative disease (Johri and Beal, 2012), live imaging of axonal transport *in vivo* has been previously described for mitochondria (Bilsland et al., 2010; Bolea et al., 2014; Misgeld et al., 2007). However, it is also apparent that an impairment of mitochondrial dynamics is not *per se* necessary or sufficient to trigger neuronal death in all neurodegenerative diseases. For example, it has been shown that reversing mitochondrial trafficking deficits does not affect neuronal death and disease progression in a mouse model of ALS (Zhu and Sheng, 2011). In addition, the axonal transport of several other cargoes has been reported to be affected in neurological diseases, including signalling endosomes, autophagosomes, RNA and lysosomes (Millecamps and Julien, 2013). Importantly, the axonal transport of these cargoes has been shown to be distinct from that of mitochondria and differentially regulated (Gibbs et al., 2015; Zala et al., 2013), highlighting the importance of studying their *in vivo* axonal transport in detail.

Past *in vivo* imaging of the axonal transport of signalling endosomes has been limited to non-invasive approaches using magnetic resonance imaging (Jouroukhin et al., 2013) or whole body fluorescence imaging (Schellingerhout et al., 2009). Such techniques do not allow for the analysis of axonal transport in specific neuronal types, nor permit the real-time visualisation of individual axons and endosomes. These shortcomings greatly limit the quantitative analysis of *in vivo* axonal transport and the use of these approaches for the evaluation of new therapeutic agents aimed at normalising axonal transport in disease models.

Here, we describe the *in vivo* imaging of the axonal transport of single endosomes in motor and sensory neurons of the sciatic nerve in live anaesthetised adult mice. Labelling of endosomes is achieved using one of two fluorescently tagged probes—the atoxic binding fragment of tetanus neurotoxin ($H_CT$) (Bercsenyi et al., 2014; Bilsland et al., 2010; Bohnert and Schiavo, 2005; Deinhardt et al., 2006) or an antibody directed against the extracellular domain of the p75 neurotrophin receptor ($\alpha$-p75$^{NTR}$) (Deinhardt et al., 2007). $\alpha$-p75$^{NTR}$ allows the labelling of endosomes within p75$^{NTR}$-expressing cells, including sensory neurons and developing or stressed motor neurons (Ibanez and Simi, 2012; Xie et al., 2003).

We outline the protocol for injection of these fluorescently tagged probes into: (1) the tibialis anterior (TA) and gastrocnemius muscles (GC) of the hindlimb, allowing labelling of both motor and sensory axons of the sciatic nerve; and (2) the footpad, allowing for the specific labelling of sensory neurons. Finally, we discuss methods for the detailed analysis of axonal transport characteristics.

## 2. Materials and methods

### 2.1. Reagents

The following reagents are required:

BL21(DE3)pLys *Escherichia coli* bacteria (Agilent Technologies, cat. no. 230134), pGEX-4T3 vector (GE Life Sciences, cat. no. 28-9545-52), isopropyl ß-D-1-thiogalactopyranoside (IPTG; Sigma Aldrich, cat. no. I5502), phosphate buffered saline (PBS; Sigma Aldrich, cat. no. P4417), Tween® 20 (Sigma Aldrich, cat. no. P9416), phenylmethylsulfonyl fluoride (PMSF; Fluka-Sigma Aldrich, cat. no. 78830), benzamidine hydrochloride hydrate (Fluka-Sigma Aldrich, cat. no. 12073), glutathione-agarose (Sigma Aldrich, cat. no. G4510), human thrombin (Sigma Aldrich, cat. no. T6759), Bradford protein assay (Bio-Rad, cat. no. 500-0006), tris-(2-carboxyethyl)phosphine hydrochloride (TCEP; Thermo Scientific, cat. no. 20490), dimethyl sulfoxide (DMSO; Sigma Aldrich, cat. no. 41648), AlexaFluor555$C_2$ maleimide (Life Technologies, cat. no. A-20346), AlexaFluor647 antibody labelling kit (Life Technologies, cat. no. A-20186), recombinant human BDNF (50 ng/$\mu$l in distilled water; Peprotech, cat. no. 450-02), isoflurane (National Veterinary Services, UK), 70% ethanol solution (v/v in distilled water), saline (0.9% NaCl w/v).

### 2.2. Equipment and software

The following equipment (or similar alternatives) is required: Superdex 200HR gel filtration column (GE Healthcare, cat. no. 17-1088-01), Amicon Ultra, Ultracel-30K filter device (Millipore, UFC803024), PD10 desalting column (GE Healthcare, cat. no. 17-0851-01), Amicon Ultra-0.5 filter device (Millipore, UFC505096), Nanodrop spectrophotometer (Labtech International), Zeiss LSM 780 NLO Multiphoton-prepared confocal microscope, oil immersion 40× objective (Zeiss EC PlnN 40×/1.3 Oil DIC II), oil immersion 63× objective (Zeiss Plan Apo 63×/1.4 Oil DIC II), immersion oil Immersol™ 518 F fluorescence free (cat. no. 444960-0000-000), tuned damped optical table (Smart Table ST-UT2 Series, Newport), environmental chamber (Zeiss XL multi S1 DARK LS; cat. no. 411857-9420-000), computer with microscope control and image acquisition software (Zen System 2012, cat no. 410135-1003-120), anaesthetic chamber and mask, hair clipper, cotton swabs, thermal blanket (such as a recirculating warm water or infrared heating pad), Zeiss Opmi MDU operating microscope with stand, surgical tape (3M™ Micropore™, cat. no. 1530S-1), scalpel (Swann Morton no. 15 disposable scalpels, cat. no. 0505), microsyringe (Hamilton, model 701 RN, 26s gauge/51 mm; cat. no. 80330), sterile suture material (PDS™ II 6-0, 45 cm length, needle 3/8 circle; Ethicon), curved forceps (John Weiss International, Jewellers Forceps, no. 7, curved, cat. no. 0101374), straight forceps (John Weiss International, Jewellers Forceps, no. 5 straight, very fine, no. 0101472), scissors, Parafilm®, microscope stage for imaging of small rodents (custom made for Zeiss LSM 780; Digital Pixel), glass coverslips (VWR International, 22 mm × 64 mm, thickness 1.0, cat. no. 631-0142).

Time-lapse images were analysed using ImageJ software for kymograph generation (http://imagej.nih.gov/ij/download.html) and Kinetic Imaging for tracking of individual endosomes (other similar motion analysis software can also be used).

### 2.3. Equipment setup

The protocol utilises an inverted confocal microscope (Supplementary Fig. 1) equipped with a 40× EC Plan-Neofluar oil immersion objective with a numerical aperture (NA) of 1.3 and a

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