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Abstract

We describe a strategy for fluorescent imaging of organelle transport in primary hippocampal neurons treated with amyloid-β (Aβ) peptides that cause Alzheimer’s disease (AD). This method enables careful, rigorous analyses of axonal transport defects, which are implicated in AD and other neurodegenerative diseases. Moreover, we present and emphasize guidelines for investigating Aβ-induced mechanisms of axonal transport disruption in the absence of nonspecific, irreversible cellular toxicity. This approach should be accessible to most laboratories equipped with cell culture facilities and a standard fluorescent microscope and may be adapted to other cell types.

INTRODUCTION

FAST AXONAL TRANSPORT IN HEALTHY AND DISEASED NEURONS

Microtubule-based intracellular transport is required by all eukaryotic cells for proper spatiotemporal delivery of proteins and organelles. Intracellular transport is particularly critical for neurons due to their extreme morphological dimensions, polarity, and need for efficient communication between the cell body and distal processes (Craig & Banker, 1994). Cytosolic proteins and cytoskeletal proteins, such as neurofilament subunits and tubulin, are moved from the cell body by slow axonal transport. In contrast, large membranous organelles are moved to and from the axon terminals by fast axonal transport (FAT). Because the axon is largely devoid of biosynthetic machinery, it relies on FAT to supply axon terminals with neurotrophic factors, lipids and mitochondria, and to prevent accumulation of toxic aggregates by clearing recycled or misfolded proteins (Maday, Twelvetrees, Moughamian, & Holzbaur, 2014; Millecamps & Julien, 2013). Bidirectional FAT is driven by kinesin and dynein motor proteins that use ATP hydrolysis to transport cargoes anterogradely toward the synapse or retrogradely toward the cell body, respectively. Additionally, protein complexes, termed adapters, are associated with molecular motors and
regulate specific cargo interactions by integrating extracellular and intracellular signals (Maday et al., 2014).

Substantial evidence implicates defective FAT in several neurodegenerative diseases, including Alzheimer’s disease (AD), and has been reviewed extensively (Goldstein, 2012; Hinckelmann, Zala, & Saudou, 2013; Maday et al., 2014; Millecamps & Julien, 2013). Damage to axonal transport in these diseases typically involves disruption of motor—cargo binding or motor—microtubule interactions. Many studies support a causal role for FAT disruption in AD (Lazarov et al., 2007; Morihara et al., 2014; Pigno et al., 2003; Stokin et al., 2005). Impaired trafficking precedes late-stage hallmarks of AD, such as amyloid plaque deposition and extensive aggregation and hyperphosphorylation of the microtubule-associated protein, tau (Minoshima & Cross, 2008; Ramser et al., 2013). Axonal swellings indicative of FAT disruption are found in postmortem samples from early sporadic AD patients (Stokin et al., 2005). Finally, using genetic and functional approaches, we detected increased production of amyloid-β (Aβ) peptides that cause AD in transgenic mice and in patients that overexpress a variant of kinesin light chain (Morihara et al., 2014). This result strongly suggests that intracellular trafficking underlies AD.

**Aβ Oligomers Impair Axonal Transport in AD**

Aβ peptides are generated when the amyloid precursor protein (APP) is cleaved sequentially by β- and γ-secretases and undergoes a toxic gain of function (Querfurth & LaFerla, 2010). Substantial evidence suggests that the soluble, oligomeric form of Aβ (AβO) accumulates in AD brain prior to detectable formation of Aβ plaques and tau tangles (Nishitsuji et al., 2009; Tomiyama et al., 2010) and is the most potent neurotoxin in AD (Ferreira & Klein, 2011). AβOs cluster at synapses, where they are thought to interact preferentially with postsynaptic membrane receptors at dendritic spines and modulate their activity (Cochran, Hall, & Roberson, 2014). Chronic AβO exposure leads to endocytic internalization of glutamate receptors, culminating in dendritic spine retraction, synapse deterioration or elimination, loss of synaptic plasticity, and cognitive deficits. In addition to their deleterious effects at synapses, AβOs activate kinase signaling cascades that cause general neuronal dysfunction (Ferreira & Klein, 2011; Krafft & Klein, 2010). Such impacts include calcium dyshomeostasis, proteasome inhibition and ER stress, oxidative stress and mitochondrial damage, aggregation and hyperphosphorylation of tau, and impairment of FAT. Activated kinases may instigate late transport defects that arise from tau hyperphosphorylation and microtubule dissolution. Alternatively, these kinases may target motor proteins and cargo adapters prior to severe tau pathology to induce early transport defects (Moreini, Szebenyi, Elluru, Ratner, & Brady, 2002; Shaw & Chang, 2013; Weaver et al., 2013). Our laboratory investigates the latter phenomenon, which precedes cell death and contributes to AD pathogenesis (Gan & Silverman, 2015; Ramser et al., 2013; Takach, Gill, & Silverman, 2015). Consequently, we design
our experimental conditions to simulate early AD and minimize nonspecific, irreversible cellular toxicity.

**APPROACHES TO IMAGING AXONAL TRANSPORT IN MODELS OF AD**

Axonal pathologies indicative of FAT disruption, such as swellings and spheroids, were initially observed by histological staining in fixed samples of human AD brain (Masliah et al., 1993; Stokin et al., 2005). Here, we discuss several live imaging approaches to evaluate the dynamic properties of normal and impaired axonal transport in invertebrate and mammalian systems.

**INVERTEBRATE TECHNIQUES**

Advances in differential interference contrast (DIC) and fluorescence microscopy have permitted high-resolution imaging of organelles in extruded squid axoplasm, *Drosophila melanogaster*, and *Caenorhabditis elegans*. Permeability barriers, such as the plasma membrane and a connective tissue sheath containing glial cells, impede external manipulation and complicate analyses of intracellular transport in the axon. It is possible to mechanically extrude a cylinder of axoplasm from the squid giant axon, effectively separating it from the plasma membrane and connective tissue sheath (Brady, Lasek, & Allen, 1985; Brady, Richards, & Leopold, 1993). Membrane-bound organelles (MBOs) within the axoplasm are readily imaged by video-enhanced contrast differential interference contrast (VEC-DIC) microscopy. Because extruded axoplasm maintains its structural integrity and metabolic activity in vitro and permits precise control of experimental conditions, it has served as a model system for decades and has yielded many seminal discoveries in the field of axonal transport and neurodegeneration. Injection of Aβ peptides into the axoplasm permits investigation of downstream signaling cascade modulation and MBO transport disruption (Pigino et al., 2009). Expression of human tau in squid axoplasm perturbs MBO transport, validating the examination of human disease proteins and pathologic mechanisms in this invertebrate model system (Kanaan et al., 2011). Furthermore, motor protein immunoprecipitation and treatments with kinase inhibitors have implicated phosphorylation as a key regulator of motor–cargo and motor–microtubule binding (Morfini et al., 2004, 2002). Despite these advantages, white light DIC microscopy only detects bulk MBO transport in the axon, and it is not possible to identify and image individual organelles, such as dense-core vesicles, synaptic vesicle precursors, and mitochondria.

*Drosophila* is an advantageous model system for in vivo transport analyses because it is amenable to genetic manipulations that identify specific organelles, such as mitochondria and APP vesicles, and mimic disease states. Transgenes encoding fluorescent-tagged organelle markers are readily integrated into the *Drosophila* genome by various techniques, including P-element, Cre-loxP, and FC31 insertion.
Transgenic third instar larvae are dissected and mounted, and axonal transport is recorded by confocal microscopy (Levitan, Lanni, & Shakiryanova, 2007). To study FAT defects, Drosophila strains carrying human AD mutations are crossed with strains expressing fluorescent organelle markers. This model system has yielded many important discoveries in APP transport and regulation. An early study reported that deletion of APP or overexpression of human APP mutations in Drosophila causes FAT defects (Gunawardena & Goldstein, 2001). More recent work revealed that glycogen synthase kinase 3β (GSK3β), a central enzyme in AD pathogenesis, phosphorylates and inhibits kinesin-1 and cytoplasmic dynein (Weaver et al., 2013). This negative regulation is accomplished by reducing the number of motors bound to microtubules. Upstream signaling cascades that modulate GSK3β are also studied extensively in Drosophila (Dolma et al., 2014; Shaw & Chang, 2013). Similarly, mechanisms of APP transport impairment have been investigated by live imaging in C. elegans (Wiese, Antebi, & Zheng, 2010). Despite the utility of invertebrate model systems for transport studies, it is ultimately challenging to demonstrate that they exhibit the same spectrum of neurotoxicity and pathological mechanisms similar to those observed in human disease.

**MAMMALIAN IN VIVO TECHNIQUES**

In vivo pulse radiolabeling can be used to analyze bulk axonal transport rates in the primary optic pathway of adult mice (Yuan, Kumar, Peterhoff, Duff, & Nixon, 2008; Yuan, Kumar, Sasaki, Duff, & Nixon, 2013). Newly synthesized proteins in retinal ganglion cells are radiolabeled by intravitreal isotope injection. At various time points thereafter, optic pathways from injected animals are dissected into consecutive segments and analyzed by SDS-PAGE, electrotransfer of proteins, phosphorimaging, and autoradiography. The distance traveled by the labeled proteins away from the injection site between time points is measured to calculate transport rates. This technique is advantageous for providing in vivo transport data from various genetic mouse models of disease. Although it is possible to distinguish slow transport of neurofilaments from fast transport of membranous organelles, this method does not enable high-resolution imaging of specific organelles. Furthermore, longitudinal studies cannot be performed in this system because animals must be sacrificed for analysis at each time point.

To circumvent this challenge, manganese (Mn²⁺)-enhanced magnetic resonance imaging (MEMRI) may be employed. Originally developed for anatomical studies and pathway tracing in the brain, MEMRI also enables assessment of axonal transport in living mice due to the unique properties of Mn²⁺. As a Ca²⁺ analog, Mn²⁺ enters neurons through voltage-gated Ca²⁺ channels, flows into the endoplasmic reticulum, and ultimately leaves in MBOs that undergo microtubule-based transport (Inoue, Majid, & Pautler, 2011). MEMRI has predominantly focused on axonal transport in the fascicles of the olfactory bulbs, and recent work identified a deficit in olfactory bulb axonal transport in aged and AD model mice (Kim, Choi,
Michaelis, & Lee, 2011; Smith, Paylor, & Pautler, 2011). Significantly, FAT defects were observed by MEMRI in APP<sub>swg</sub>/PS1<sub>dE9</sub> mice prior to Aβ plaque formation and extensive tau hyperphosphorylation (Minoshima & Cross, 2008). Noninvasive MEMRI studies can be performed repeatedly on live animals and will enable longitudinal measurements of FAT throughout AD progression, unlike traditional approaches using radiolabeling and cell tracer dyes (Inoue et al., 2011).

MAMMALIAN IN VITRO TECHNIQUES

In vitro live imaging techniques were developed to study the dynamic properties of specific cargoes and molecular mechanisms of transport in single neurons, which cannot be obtained by electron microscopy on fixed samples, biochemical dissection of labeled materials, or in vivo techniques. High-resolution imaging of organelles is performed in mammalian model systems such as cell lines, primary neurons, and human neurons derived from induced pluripotent stem cells. Here, we discuss the advantages and shortcomings of each experimental paradigm.

To assess transport of specific organelle populations in healthy or AD states, fluorescently transfected into nonneuronal cell lines like PC12 or neuronal cell lines such as SH-SY5Y or CAD. Overexpression of hyperphosphorylated tau expands intermicrotubule spacing and inhibits mitochondrial movement in the neuritic processes of PC12 cells (Shahpasand et al., 2012). In SH-SY5Y cells that acquire neuronal morphology and function through differentiation, proteosome inhibition activates stress kinases implicated in tau phosphorylation and AD pathology, impairing transport of vesicles and mitochondria (Agholme et al., 2014). Moreover, in brainstem-derived CAD cells, a persistent stress response to impaired transport of APP vesicles leads to accumulation of Aβ in the ER and may contribute to sporadic AD (Muresan & Muresan, 2012). Although these studies demonstrate the utility of cell lines for expression of specific cargo markers and investigation of transport dynamics and regulation, cell lines may not faithfully recapitulate in vivo neuronal physiology and disease states.

Primary neurons and cell lines possess similar experimental advantages; however, primary neurons are superior because they are highly polarized, enabling clear identification of axons and dendrites and compartment-specific analyses of FAT. The uniform, plus-end-out organization of axonal microtubules permits efficient analysis of anterograde and retrograde transport driven by canonical kinesin and dynein motors, which move cargo in opposite directions (Baas, Deitch, Black, & Banker, 1988). Importantly, transport defects can be examined in primary neurons cultured from genetic models of AD, such as triple transgenic mice (3×Tg), which carry mutations in APP, presenilin, and tau and exhibit premature deficits in spatial learning and memory (Oddo et al., 2003). Impaired FAT of mitochondria, neuropeptide vesicles, and synaptic vesicle precursors has been extensively reported in wild-type primary neurons treated with exogenous Aβ (Bomfim et al., 2012; Gan & Silverman, 2015; Guo et al., 2013; Hiruma, Katakura, Takahashi, Ichikawa, & Kawakami, 2003; Poon et al., 2013; Ramser et al., 2013; Tang et al., 2012; Vossel et al., 2010). Transgenic rodents are a
tractable model system with a long history of physiological, cellular, and biochemical techniques applied to multiple brain regions and neuron classes. Although rodent models have provided important insights into AD, studies are often confounded by overexpression artefacts, and mutations introduced into endogenous genes fail to recapitulate all phenotypes and behaviors associated with human AD pathology (Duff & Suleman, 2004; Young & Goldstein, 2012).

Recent advances in reprogramming technology have enabled the expression of defined factors in somatic cells, such as skin fibroblasts, from an individual patient to induce a pluripotent stem cell state (iPSC; Takahashi et al., 2007). These iPSCs can be differentiated into neurons that retain the unique genetic background of the individual. Alternatively, patient fibroblasts can be exposed to forebrain transcription factors that directly convert them to neurons (induced neuronal (iN) cells). iPSC and iN models of AD are derived from presenilin and APP point mutations and APP gene duplications associated with familial forms of AD (Israel et al., 2012; Kondo et al., 2013; Yagi et al., 2011). Sporadic AD genomes confer similar

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Reporter Protein</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>Ds-Red2-Mito, Mito-eYFP, Mito-eGFP</td>
<td>Clontech, Addgene #56596, Llopis, McCaffery, Miyawaki, Farquhar, &amp; Tsien (1998)</td>
</tr>
<tr>
<td>Dense core vesicle</td>
<td>BDNF-mRFP/GFP, ChAr-eGFP/RFP, NPY-eGFP, tPA-eGFP</td>
<td>Courel et al. (2006), Haubensak, Narz, Heumann, &amp; Lessmann (1998), Lochner et al. (2008), Mukherjee et al. (2011), Silverman et al. (2005)</td>
</tr>
<tr>
<td>Synaptic vesicle precursors and PTVs</td>
<td>Synaptophysin-GFP, VAMP-GFP, mRFP-Bassoon, ELKS2-iEGFP</td>
<td>Ahmari, Buchanan, &amp; Smith (2000), Maas et al. (2012), Sun &amp; Bamji (2011)</td>
</tr>
<tr>
<td>APP-containing vesicle</td>
<td>CFP-APP-YFP, APP-YFP, APP-mCherry/GFP</td>
<td>Rodrigues, Weissmiller, &amp; Goldstein (2012), Villegas, Muresan, &amp; Ladescu Muresan (2014)</td>
</tr>
<tr>
<td>BACE-containing vesicle</td>
<td>eYFP-BACE1, mCherry/GFP-BACE1</td>
<td>Buggia-Prevot et al. (2014), Das et al. (2013)</td>
</tr>
<tr>
<td>Autophagosome</td>
<td>tflLC3, LC3-eGFP, mRFP-LC3</td>
<td>Kimura, Noda, &amp; Yoshimori (2007), Mizushima, Yamamoto, Matsui, Yoshimori, &amp; Ohsumi (2004), Addgene #21074, 21075</td>
</tr>
<tr>
<td>Lysosome</td>
<td>LAMP1-mGFP</td>
<td>Addgene #34831</td>
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<tr>
<td>Peroxisome</td>
<td>eGFP-SKL</td>
<td>Das et al. (2013), Kim et al. (2007)</td>
</tr>
<tr>
<td>Endosomes/signaling endosomes</td>
<td>pAcGFP1-Endo, GFP-EEA1, TrkB-GFP</td>
<td>Clontech, Addgene #42307, Watson et al. (1999)</td>
</tr>
<tr>
<td>Post-Golgi vesicles</td>
<td>NgCAM, Tr-VAMP</td>
<td>Burgo et al. (2012), Sampo, Kaech, Kunz, &amp; Banker (2003)</td>
</tr>
</tbody>
</table>

Table 1 Fluorescent Chimeric Proteins to Visualize Organelles
cellular phenotypes: neurons from a sporadic AD patient exhibit elevated levels of Aβ, calcium dyshomeostasis, increased activity of AD-related kinases, and tau hyperphosphorylation compared to neurons from age-matched, normal individuals (Israel et al., 2012). This model system is ideal for high-resolution imaging of specific organelle transport and detection of spatiotemporal changes in protein localization. Although impaired FAT has been reported in neurons derived from amyotrophic lateral sclerosis, Charcot–Marie–Tooth disease, and hereditary spastic paraplegia disease patients (Alami et al., 2014; Denton et al., 2014; Saporta et al., 2015), transport defects have not been detected and characterized in human stem cell models of AD.

**AβO SOURCES AND PREPARATIONS**

For experimental purposes, AβOs are derived from three primary sources: purification from AD brain, production of “naturally secreted” oligomers from a cell line, and oligomerization of synthetic peptides. We use synthetic AβOs in our studies for several reasons. First, they mimic the toxic properties of natural oligomers (brain or cell derived) as described previously (Ferreira & Klein, 2011; Jin et al., 2011; Welzel et al., 2014). Second, amino acid substitutions may be introduced to reduce toxicity, and scrambled peptides may be generated as controls (Decker, Lo, Unger, Ferreira, & Silverman, 2010; Fonte et al., 2011). Finally, unlike natural oligomers, synthetic AβOs can be detected by immunocytochemistry or labeled directly for live cell imaging (Figure 2(A)). Confirmation of AβO binding is crucial in our experiments because it varies considerably between neurons. Although they may not be identical to natural oligomers, synthetic AβOs are a tractable tool for investigating mechanisms of AD pathogenesis. We follow a universal protocol for preparation of synthetic AβOs (Lambert et al., 1998; Renner et al., 2010), and we are in contact with Dr W. Klein’s (Northwestern University) laboratory to stay informed of any modifications. Although synthetic oligomers are typically used at a higher concentration than what is found in AD brain, they elicit similar cellular pathologies as described previously (Ferreira & Klein, 2011; Jin et al., 2011). With proper controls for viability, experiments can be performed over a wide range of AβO concentrations. At higher AβO concentrations, a rapid onset of toxicity might mask subtler defects, such as signaling cascade dysregulation, that precede microtubule dissolution and cytoskeletal collapse.

**LIVE IMAGING OF ORGANELLES IMPLICATED IN AD**

Most studies on FAT impairment in AD have focused on mitochondria, vesicles containing APP and/or β-secretase (BACE), and vesicles containing neurotrophic factors (Almenar-Queralt et al., 2014; Das et al., 2013; Decker, Lo, et al., 2010; Hiruma et al., 2003; Poon et al., 2013; Vossel et al., 2010).
mitochondria can be broadly applied to investigate mechanisms of FAT regulation for other cargoes implicated in AD, such as synaptic vesicle precursors, endosomes, and autophagosomes. Below, we suggest reliable fluorescent-tagged markers for specific organelles that are commercially available or obtained from established laboratories. It is advisable to use confocal microscopy to detect cargoes that accumulate on the plasma membrane, including NgCAM, VAMP, and synaptophysin; otherwise, wide-field fluorescent microscopy suffices. Although organelle dyes, such as MitoTracker and LysoTracker (Life Technologies), are widely used, we find that they are not ideal for transport studies on Banker-style primary cultures (see Section 1). Well-isolated neurites are essential for tracing cargo dynamics back to individual cells; even at low density, a mature culture possesses many axons and dendrites that are readily stained by organelle dyes and overlap extensively.

1. METHODS

In this section, we describe a strategy for culturing primary neurons, transfecting them with fluorescently labeled organelle markers, preparing synthetic AβOs and applying them to transfected cultures, and measuring axonal transport (Figure 1). Our method enables careful, rigorous analyses of axonal transport defects, which are implicated in AD. Importantly, we present and emphasize guidelines for investigating AβO-induced transport defects in the absence of cellular toxicity.

1.1 PRIMARY NEURONAL CULTURE

We prepare primary dissociated neurons from E18 rat or E16 mouse hippocampi as described by Kaech and Banker (2006). Briefly, neurons are cultured at low density on poly-L-lysine-treated coverslips, which are suspended above an astrocyte feeder layer and maintained in serum-free medium. For live imaging studies, we typically plate cells at low density (2 × 10^5 cells per 6-cm dish, each containing four to five coverslips). When cultured according to this protocol, hippocampal neurons become polarized, develop extensive axonal and dendritic arbors, and form functional synaptic connections. Banker-style cultures are widely used for visualizing protein

FIGURE 1 Timeline of imaging experiments.
transport and subcellular localization and defining molecular mechanisms that regulate trafficking, polarity, dendritic growth, and synapse formation. Preparation of the astrocyte feeder cultures must begin 2 weeks in advance, and it takes 5 days to prepare coverslips as a substrate for neuronal growth. Dissecting hippocampi and plating dissociated neurons takes 2—3 h.

1.2 TRANSFECTIONS

In addition to an organelle marker, neuronal transfections should include a soluble marker for initial identification of processes based on morphological criteria and determination of axonal orientation relative to the cell body (Figure 2(A)). A variety of lipid-based reagents, such as Lipofectamine 2000 (Invitrogen), have been developed for transfecting plasmids into cultured cells, and several of these are also effective for primary neurons. Although most give high levels of expression, the transfection efficiency is comparatively low (0.1—5%). In our hands, the transfection efficiency varies over the lifetime of the culture; between 8 and 10 days in vitro (DIV), approximately 1—3% of the neurons express the transgene, which is ideal for our live imaging studies.

1.2.1 Transfection protocol (Reproduced from Kwinter & Silverman (2009))

1. For each transfection, label two 1.5-mL tubes: one to contain the plasmid DNA, and the other to contain the transfection reagent. The following incubations can proceed at room temperature:

   a. Combine 1.0 μg of plasmid DNA with 100 μL MEM in one tube (without supplements of any type; MEM does not need to be temperature- or pH-equilibrated).

   b. Combine 6.0 μL Lipofectamine with 100 μL MEM in the other 1.5-mL tube. Note: No adjustments for double transfections are required, even though the Lipofectamine:DNA ratio will be halved in such cases. The ratio of Lipofectamine:DNA is still within the suggested range. It is best to test 0.5—1.0 μg for each plasmid for optimal expression. To preserve the shelf life of the Lipofectamine reagent, it should be removed from the refrigerator for as short a time as possible and placed in a benchtop cooler when not in use. Total time out of the refrigerator should be kept to a minimum.

2. Incubate the tubes for 5 min at room temperature.

3. Transfer the DNA-in-MEM solution into the lipid tube, and gently mix with a pipette. Incubate for 30 min at room temperature.

4. After 25 min, add 60 μL of 50 mM kynurenic acid before flipping coverslips to minimize excitotoxic damage to the dish of cultured neurons.

Note: Carefully flip coverslips feet-side-up using sterile forceps and arrange them so they do not overlap. Be careful not to scrape the neuron-coated surface of the coverslip or the glia-coated surface of the dish.
5. Take approximately 0.5 mL of medium from the 6-cm dish and gently mix with DNA in tube, transfer the DNA solution back to the dish, and drip it evenly onto surface of the medium.

6. Do not swirl, but gently slide dish back and forth in one direction, then stop and repeat in perpendicular direction to evenly distribute the DNA-Lipofectamine.
complexes. Incubate for 90 min in the tissue culture incubator (37 °C, 5% CO₂).

7. Flip coverslips feet-side-down, and arrange so they do not overlap. Allow expression to proceed for the desired time, typically overnight to 48 h.

1.3 **AβO PREPARATION AND APPLICATION**

Amyloidogenic cleavage of APP releases two major monomeric Aβ isoforms: Aβ₁₋₄₀ predominates and remains soluble; however, Aβ₁₋₄₂ is more prone to aggregation and is thus the major constituent of oligomers, fibrils, and plaques in AD patients. We use the following Aβ₁₋₄₂ peptides to prepare synthetic AβOs: unmodified peptides (1 mg, American Peptide, Sunnyvale, CA) and HiLyte-488 or HiLyte-555 fluorescent peptides (0.1 mg, AnaSpec, San Jose, CA), prepared in trifluoroacetic acid (TFA). We highly recommend these sources; in our experience, oligomer yield and stability were compromised when peptides were purchased elsewhere or prepared using other solvents. Below, we describe the monomerization of Aβ peptide stocks, storage of Aβ films, and subsequent preparation of AβOs (adapted from Lambert et al. (1998) and Renner et al. (2010)).

1.3.1 **Monomerization and storage of unmodified and fluorescent Aβ peptides**

1. Solid Aβ peptide stocks are stored at −80 °C. Remove and place on ice when ready to prepare stock peptide films.

2. Place 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP, Sigma H-8508) on ice in the hood to cool. HFIP is highly corrosive and volatile. Add 221.5 μL of cold HFIP to the original vial of unmodified Aβ and 205.3 μL to the original vial of fluorescent Aβ to obtain a final peptide concentration of 1 and 0.1 mM, respectively. Rinse vials thoroughly.

   Note: Removal of any preexisting structures in solid Aβ stocks is critical for controlled aggregation studies. HFIP disrupts hydrophobic forces and breaks down β-sheet structures in aggregated amyloid preparations, yielding a dense, homogenous solution of monomers.

3. Incubate closed vials at room temperature for at least 1 h. Although peptide preparations might appear milky at first, they should clear within 5 min. Preparations that remain cloudy indicate poor peptide quality and should not be used.

4. Place the peptide-HFIP solutions back on ice for 10 min.

5. Aliquot the peptide solutions into nonsiliconized microcentrifuge tubes, typically 5 μL (22.6 μg peptide) per tube for unmodified Aβ or 11.6 μL (5.7 μg peptide) per tube for fluorescent Aβ.

6. Leave tubes open in the hood. Allow HFIP to evaporate overnight at room temperature.

7. Transfer tubes to a SpeedVac and dry down for 10 min. All traces of HFIP must be removed. The resulting peptide should be a thin, colorless film for
unmodified Aβ, a red film for HiLyte-488 Aβ, or a pink film for HiLyte-555 Aβ at the bottom of the tubes. The peptides should not appear opaque or chunky.

8. Store the dried Aβ films over a desiccant at −80 °C. These stocks are stable for several months.

1.3.2 Preparation and application of unmodified and fluorescent Aβ oligomers

Note: AβOs must be prepared fresh. Do not store the resuspended peptide for longer than 2–3 days at 4 °C, as protofibrils will form. Begin the following procedure two days before to live imaging (Figure 1).

1. Remove peptide films from −80 °C freezer and place on ice.
2. To prepare unmodified AβOs, dissolve peptide film in 1.0 μL anhydrous DMSO (Sigma). Pipette thoroughly, washing down the sides of the tube to ensure that the film has dissolved completely. Fine droplets along the sides of the tube should no longer be visible.
3. Add 49.0 μL PBS to a final unmodified AβO concentration of 100 μM.
4. To prepare fluorescent AβOs, dissolve HiLyte-488 or HiLyte-555 peptide film in 15.1 μL DMSO as described in Step 2. Transfer this solution into a tube of unmodified Aβ and again proceed as in Step 2 until the film is completely dissolved.
   Note: To prepare fluorescent AβOs, HiLyte-488 or HiLyte-555 Aβ peptides are mixed with unmodified Aβ peptides at a molar ratio of 1:4.
5. Add 57.9 μL PBS to the mixture from Step 4, yielding a final AβO concentration of 85 μM.
6. Pipette several times to mix and incubate preparations at 4 °C for 24 h.
7. Prepare a DMSO/PBS control in a sterile tube, using the same DMSO:PBS ratio as above. Incubate overnight at 4 °C for 24 h to permit oligomerization.
   Note: A scrambled AβO peptide may also be prepared as a control (Decker, Lo, et al., 2010).
8. Centrifuge tubes at 14,000 × g for 10 min at 4 °C. Carefully transfer the supernatant, which contains the AβOs, into a sterile tube without disturbing the pellet.
   Note: Do not vortex AβOs once they are prepared, as this produces protofibrils and fibrils. Gently invert the tube to mix if necessary.
9. Perform a Bradford Assay (Bio-Rad) with a BSA standard to determine the concentration of AβOs. A typical yield is 50–70 μM.
10. Apply AβOs to 10–12 DIV neurons at a final concentration of 500 nM in the medium. Incubate for 18 h in the tissue culture incubator (37 °C, 5% CO2).

1.4 LIVE IMAGING (ADAPTED FROM KWINTER & SILVERMAN (2009))

Our approach to imaging FAT in primary neurons relies on a standard wide-field fluorescent microscope outfitted with a CCD camera, image capture software, and a heated imaging chamber. We have imaged a variety of organelles or structures,
including dense-core vesicles, mitochondria, growth cones, and actin, without any special optics or excitation requirements other than a fluorescent light source. Additionally, with the use of a beam splitter and appropriate filters, spectrally distinct, fluorescently labeled proteins such as GFP and dsRed can be visualized simultaneously to characterize cotransport or other coordinated cellular events. The imaging approach described here is flexible for a variety of imaging applications and can be adopted by a laboratory for relatively little cost, provided a microscope is available.

1.4.1 Equipment setup
Imaging of live cells requires a fluorescence microscope equipped with a CCD camera; we use a Leica DMI 6000B inverted microscope equipped with the Leica variable, fluorescent lamp. Also required is an imaging chamber with temperature controller and an objective heater. We use the Warner Instruments RC-21BR modified for an 18-mm coverslip in addition to a platform heater (cat. #PH2) and a temperature controller (cat. #TC324B). The chamber contains no open ports, a modification that can be included when ordering the chamber. An objective heater is highly recommended to help maintain the temperature of the coverslip and minimize changes in focus due to temperature fluctuations. Images are acquired with a Hamamatsu Orca-ER using Metamorph (Molecular Devices) software including the “streaming” mode of acquisition drop-in. We do not use a climate chamber that encloses the entire microscope. This addition is expensive and not necessary for the short-term imaging described here. Other equipment and reagents include freshly prepared live imaging medium (1X Hanks with Ca$^{2+}$ and Mg$^{2+}$, 0.6% glucose, and 10 mM HEPES), additional 18-mm glass coverslips, sterile forceps, nonsterile forceps, Kimwipes, vacuum grease, and a syringe (without needle or with modified wide-bore needle) with which to apply grease.

1.4.2 Preparation of imaging chamber
1. Prepare fresh live imaging medium. It is best to prepare enough for no more than 1 week at a time. Approximately 5–10 mL are used per dish of neurons.
2. Start microscope, camera, fluorescent lamp, and image acquisition software. Also turn on power to the chamber platform and objective heaters.
3. Prepare the imaging chamber.
   a. The Warner chamber includes a Teflon insert for the mounting of coverslips. For ease of manipulation, label one side of the insert “top” with a permanent marker.
   b. Apply a ring of grease to the groove surrounding the hole in the side of the chamber labeled “top.” Avoid using excess as it will enter the chamber and reduce the observable area.
   c. Using forceps, attach a clean, unused 18-cm coverslip to the “top” side of the chamber, apply gentle pressure to the coverslip at its edges to create a tight seal within the recessed groove of the chamber.
Turn the chamber over and apply a similar ring of grease to the groove on the opposite (bottom) side of the chamber.

Place the prepared chamber—bottom side up—on the lid of a 50-mL tube, which is an ideal chamber holder.

Add 750 μL of imaging medium to the chamber. This is an excessive amount, but the grease should prevent the medium from spilling over and the excess will help prevent bubbles from being trapped when the cell-covered coverslip is applied.

Maintain the prepared chamber in the tissue culture incubator until needed. Prolonged incubations, ~1 h should be avoided as the imaging medium will evaporate and change in composition.

### 1.4.3 Chamber assembly and live cell imaging *(Figure 3(C))*

1. Move the prepared chamber and a dish of transfected coverslips from the incubator to the tissue culture hood.
2. The final chamber assembly should be performed quickly to ensure the cells remain immersed in medium and at 37 °C continuously.
3. Using sterile forceps, carefully remove one coverslip from the transfected dish. Touch the edge of the coverslip to a Kimwipe to draw away growth medium.
4. Place the coverslip neuron-side-down onto the prepared chamber. Touch one side of the coverslip to the grease first and apply pressure around the edges to bring the coverslip flat without trapping bubbles. Excess imaging medium will spill out as expected.
5. Wipe off excess imaging medium, but be careful not to slide the coverslips out of position.
6. Move the chamber to the preheated platform heater and fasten the chamber in place.
7. Transfer the chamber to the stage.
8. To reduce photobleaching and phototoxicity, adjust the lamp to the minimum intensity necessary to find transfected cells.
9. Using a low-magnification oil objective (40×), find a vehicle- or AβO-treated transfectant. It is wise to stick to a planned search pattern to ensure maximal coverslip coverage and to avoid redundant image acquisitions, e.g., start at top left of coverslip, scan up and down, and work to the right.

   Note: AβO binding to transfectants is highly variable; although they bind frequently to the cell body, AβOs bind to axons and dendrites in only a fraction of transfectants (~25%).
10. Switch to a high-magnification oil objective (60–100×) once a desired field has been located.

   Note: Choose a flat axonal region approximately 50–100 μm away from the cell body, excluding bifurcations that lead to uneven distribution of organelles. The axonal segment should measure at least 100 μm long and should not exhibit blebbing or fasciculation.
AβO treatments and live imaging setup. (A) It is essential that AβOs remain punctate throughout an experiment. From left to right, images demonstrate 500 nM Hylite-488 AβOs bound to neuronal processes, AβO aggregation that has led to reduced cellular binding and nonspecific background, and AβOs that have fibrillized. (B) AβO binding varies considerably between transfected neurons. The transfectant on the left has little detectable binding compared to neighboring cells. (C) Warner Instruments P-2 heater platform with an RC-21B type, closed chamber modified for an 18 mm coverslip. (See color plate)
11. Use “stream acquisition” or comparable function of your imaging software to record a video of fluorescently labeled protein dynamics. Note: Adjust imaging parameters based on the transport characteristics of the organelle. For example, mitochondria movement is sporadic and infrequent, so to capture a greater number of events, acquire an image every 5–7 s for 10 min. For organelles that move continuously, increase the frequency of image acquisition to 4–5 frames/s for 30 s to capture dozens of transport events.

12. Take a phase image and any other accompanying images for later analysis and presentation.

13. Save all images before exploring the coverslip further and recording the next video.

14. Typically, three to five videos from one coverslip is considered successful. Phototoxicity and general cell health should be kept in mind as transport is reduced in damaged cells. Cell health can be monitored by observation using phase microscopy. Typically, recordings are performed for approximately 30 min per coverslip.

1.5 KYMOGRAPH GENERATION AND ANALYSIS

Live imaging observations typically consist of videos, also known as “stacks” of images, that show movement of fluorescently tagged organelles within the field of view. Accompanying each video are supporting images that illustrate orientation of the field of view with respect to the cell body, level of protein expression, and the health of the cell (Figure 2(B)). Videos of transport can be analyzed quantitatively by transformation to kymographs. Kymographs are distance–time graphs that illustrate the trajectories of individual particles. Figure 2(B) demonstrates how bidirectional movement of BDNF vesicles and mitochondria are represented as line scans within a kymograph. Organelle flux, velocity run lengths, number of stationary particles, and frequency of reversals are obtained by tracing these line scans. Several commercially available software packages have kymograph generation and tracing applications, including Metamorph. Although we prefer to identify individual particles manually, automated kymograph-tracing software is available (Mukherjee et al., 2011). We use custom software to calculate transport statistics, but similar programs available in the public domain, such as the Kymolyzer plug-in for ImageJ and one developed by De Vos and Sheetz, are also used with ImageJ (Ashrafi, Schlehe, LaVoie, & Schwarz, 2014; De Vos & Sheetz, 2007). We subsequently compile transport data and perform tests for statistical significance in Excel.

2. ANTICIPATED RESULTS AND DISCUSSION

We have described a strategy for fluorescent imaging of organelle transport in primary hippocampal neurons treated with AβOs (Figure 1). Our method is comprised
of previously established techniques that must be combined and executed meticulously and consistently to investigate pathogenic mechanisms of AβOs in the absence of cellular toxicity. It is critical to maintain healthy polarized neuronal cultures, prepare AβOs correctly, acquire transport data efficiently, and monitor cell viability during experiments. Under these conditions, our kymographs of axonal BDNF transport illustrate that a large fraction of control vesicles are mobile, moving uninterrupted in one direction, as denoted by diagonal traces (Figure 2). Stationary vesicles are depicted by horizontal lines. Transport is highly processive in both anterograde and retrograde directions. Run lengths for mitochondria are similarly high; however, their movement is characteristically sporadic and infrequent and exhibits an anterograde bias (Fang, Bourdette, & Banker, 2012; Wang & Schwarz, 2009). Organelles exhibit a range of velocities, but most traces are roughly parallel, indicating similar velocities. AβO treatment significantly reduces these transport parameters (Figure 2). We and others have shown that AβO-induced FAT defects are reversible (Gan & Silverman, 2015; Takach et al., 2015; Tang et al., 2012). This is the strongest indicator that cell health is not compromised. To complete an experiment, we analyze 15–20 cells per condition from three independent cultures, yielding hundreds to thousands of transport events. Depending on the number of conditions tested and culture frequency, a single imaging session may last up to 14 h, and it may take several weeks to complete an experiment.

2.1 COMMON PROBLEMS AND TROUBLESHOOTING SUGGESTIONS

1. **Neurons are poorly transfected**: Prepare fresh DNA and analyze it by gel electrophoresis to ensure that it is predominantly supercoiled and purchase fresh transfection reagent. In our experience, fasciculated cultures and plating neurons at higher density (>250,000 cells per dish) lead to poor transfections. Prevent excessive fasciculation by ensuring proper acid and poly-L-lysine treatments of coverslips and reducing astrocyte plating density (Kaech & Banker, 2006).

2. **High transfection efficiency but little or no detectable organelle movement**: Prepare fresh live imaging medium and prewarm it to 37 °C prior to mounting neuronal coverslips. Ensure that the temperature of the chamber remains at or slightly below 37 °C. Work quickly to load coverslips, search for transfectants, and acquire movies. To reduce photobleaching and phototoxicity, adjust the lamp to the minimum intensity necessary.

3. **Aβ fibrils or large aggregates present in the AβO preparation (Figure 3(A))**: If fluorescent oligomers are used, these undesired conformations are immediately obvious upon live imaging. They might induce cellular insults that are not specific to oligomers, dilute the effective concentration of oligomers, and obscure axonal regions. Ensure that Aβ peptides are synthesized in TFA, and that films are stored at −80 °C for no longer than 2–3 months. Solubilize Aβ films in DMSO/PBS; in our hands, F12 promotes the formation of aggregates.
AβOs must be prepared fresh. Do not store the resuspended peptide for longer than 2–3 days at 4 °C, as protofibrils will form.

4. Sparse AβO binding (Figure 3(B)): At 500 nM, AβO binding to transfectants is highly variable in our experience; although they bind frequently to the cell body, AβOs bind to axons and dendrites in only a fraction of transfectants (~25%). It is possible that lipid-based transfections transiently disrupt plasma membrane integrity and reduce cell-surface AβO binding sites. We have yet to test alternative transfection methods, such as electroporation, calcium phosphate, magnetic beads, and viral infections, for compatibility with AβO treatments and cell viability. AβO binding also varies with the age of the culture: significant binding occurs only after 9 DIV, when postsynaptic glutamate receptors begin to cluster (Rao, Kim, Sheng, & Craig, 1998). This supports the notion that AβOs at nanomolar concentrations preferentially interact with dendritic and axonal receptor proteins that are expressed in mature neurons (Decker, Jurgensen, et al., 2010; Gan & Silverman, 2015), rather than forming random pores in the plasma membrane (Benilova, Karran, & De Strooper, 2012). Moreover, we do not observe downstream Aβ pathologies, such as tau hyperphosphorylation and transport disruption, in cultures younger than 9 DIV.

2.2 ASSESSMENT OF CELL VIABILITY

It is essential to monitor and maintain neuronal health throughout this method: while culturing and transfecting the cells, treating them with AβOs, and imaging transport. The most straightforward assessment of cell viability is the phase contrast (or DIC) view. Neurons should have uniformly looking processes that are free of blebs, which appear as black beads on axons and dendrites (Figure 4(A), left). Note that Lipofectamine produces randomly distributed, black speckles that appear much smaller than membrane blebs; these are nontoxic. Additionally, cell bodies should appear rounded, yielding a “phase bright” appearance. As cell health declines, cell bodies flatten and become vacuolated (Figure 4(A), center). Ultimately, only remnants of cell body and processes remain (Figure 4(A), right). Neurons may deteriorate quite rapidly if, for example, the temperature within the imaging chamber rises beyond 37 °C. While adopting this technique, it is advisable to perform a viability assay, such as the LIVE/DEAD cytotoxicity assay (Life Technologies), to define the physiological limits of your experimental system and train your eyes to identify signs of cell deterioration. Unfortunately, we find that images of dead cells appear in the literature as representative of live cells.

We find that AβOs are not neurotoxic during the 18 h timeframe of our imaging experiments and for up to 3 days in culture (Figure 4(B)); yet, for a more rigorous assessment of cell viability beyond phase contrast appearance, immunocytochemical and biochemical methods can be employed. We have previously investigated cytoskeletal integrity by semiquantitative staining for tubulin and actin and measuring the ratio of soluble to polymerized tubulin by gentle extraction in a microtubule-stabilizing buffer (Figure 4(B)). When microtubules are destabilized,
Assessment of cell viability. (A) Phase contrast images of neurons are an immediate and reliable test of cell health. Neurons appear smooth with nonfasciculated axons and dendrites (left). Cells in the center panel have started to die, and active transport may be reduced. Although cell bodies and axons and dendrites are still visible in the right panel, these cells are
the Golgi fragments loses its perinuclear distribution. Staining for a Golgi marker such as GM130 can reveal the constitution of the secretory apparatus. Calcium-induced excitotoxicity can be detected by immunoblotting for calcineurin and caspase-3 cleavage (Figures 4(C) and (D)), and ATP production may be quantified using a luciferase-based assay (Figure 4(E)). Choose appropriate controls for your experiments as diligent reviewers will demand them.

**CONCLUSION**

Live imaging is an essential method for investigating dynamic properties of intracellular transport. FAT is of particular interest in neurobiology because it underlies fundamental processes and functions, such as polarization, synaptic protein resupply, neuropeptide secretion, and axonal maintenance. Proper regulation of FAT is therefore critical for neuronal development, communication, and survival. Growing evidence suggests that transport defects cause or exacerbate multiple neurodegenerative diseases, including AD. Thus, compounds that restore motor—cargo interactions, motor—microtubule associations, and motor protein motility are excellent candidates for therapeutic interventions. It is important to realize that mimicking a neurodegenerative disease in a dish is challenging, and experimental conditions must be carefully controlled to ensure that observed transport impairment is specific and not simply a secondary consequence of toxicity.

**ACKNOWLEDGMENTS**

We thank R.P. Vieira and B. Pasqualotto for their critical reading of this manuscript. K.J.G. is funded by a C.D. Nelson Memorial Graduate Scholarship from Simon Fraser University and...
an NSERC Postgraduate Scholarship. This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC; 327100-06) and the Canadian Institutes of Health Research (CIHR; 90396) to M.A.S.

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