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Real-time Imaging of Axonal Transport of Quantum dot-labeled BDNF in Primary Neurons

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Abstract:	BDNF plays an important role in several facets of neuronal survival, differentiation and function. Structural and functional deficits in axons are increasingly viewed as an early feature of neurodegenerative diseases, including Alzheimer's disease (AD) and Huntington's disease (HD). As yet unclear is the mechanism(s) by which axonal injury is induced. We reported the development of a novel technique to produce biologically active, monobiotinylated BDNF (mBtBDNF) that can be used to trace axonal transport of BDNF. Quantum dot-labeled BDNF (QD-BDNF) was produced by conjugating quantum dot 655 to mBtBDNF. A microfluidic device was used to isolate axons from neuron cell bodies. Addition of QD-BDNF to the axonal compartment allowed live imaging of BDNF transport in axons. We demonstrated that QD-BDNF moved essentially exclusively retrogradely, with very few pauses, at a moving velocity of around 1.06 μm/s. This system can be used to investigate mechanisms of disrupted axonal function in AD or HD, as well as other degenerative disorders.
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Question	**Response**

Dec 20, 2013

Dr Jane Hannon
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Dear Dr Jane Hannon:

Many thanks for your invitation for submitting our work for your consideration. Our manuscript is entitled: "Real-time Imaging of Axonal Transport of Quantum dot-labeled BDNF in Primary Neurons". In the manuscript, we describe a novel method for producing biologically active brain derived neurotrophic factor (BDNF) that is homogeneously labeled with a single biotin moiety (mBtBDNF). By conjugating to fluorescent nanocrystals (Quantum dots), mBtBDNF can be used for realtime tracking axonal transport of BDNF at the single molecule level in primary neurons that are cultured in microfluidic chambers.

Axonal trafficking of BDNF is critical for neuronal function and maintenance in the central nervous system. These technologies offer an excellent in vitro model and allow the characterization of the highly dynamic process of axonal transport of BDNF with ultra-spatial and temporal sensitivities. We believe that these technologies can be used to study and generate novel insights into the molecular and cellular events that lead to neurodegenerative disorders such as Alzheimer's disease, Huntington's disease.

Thank you again for your consideration. We look forward to hearing from you!

Sincerely,

Chengbiao Wu, PhD
UCSD Neurosciences

TITLE: Real-time Imaging of Axonal Transport of Quantum dot-labeled BDNF in Primary Neurons

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KEYWORDS:
Live imaging; Brain-derived neurotrophic factor (BDNF); quantum dot; trafficking; axonal retrograde transport; microfluidic chamber.

SHORT ABSTRACT:
Axonal transport of BDNF, a neurotrophic factor, is critical for the survival and function of several neuronal populations. Some degenerative disorders are marked by disruption of axonal structure and function. We demonstrated the techniques used to examine live trafficking of QD-BDNF in microfluidic chambers using primary neurons.

LONG ABSTRACT:
BDNF plays an important role in several facets of neuronal survival, differentiation and function. Structural and functional deficits in axons are increasingly viewed as an early feature of neurodegenerative diseases, including Alzheimer's disease (AD) and Huntington's disease (HD). As yet unclear is the mechanism(s) by which axonal injury is induced. We reported the development of a novel technique to produce biologically active, monobiotinylated BDNF (mBtBDNF) that can be used to trace axonal transport of BDNF. Quantum dot-labeled BDNF (QD-BDNF) was produced by conjugating quantum dot 655 to mBtBDNF. A microfluidic device was used to isolate axons from neuron cell bodies. Addition of QD-BDNF to the axonal compartment allowed live imaging of BDNF transport in axons. We demonstrated that QD-BDNF moved essentially exclusively retrogradely, with very few pauses, at a moving velocity of around 1.06 µm/s. This system can be used to investigate mechanisms of disrupted axonal function in AD or HD, as well as other degenerative disorders.

INTRODUCTION:
Neurons are highly polarized cells whose long and often highly elaborated processes are fundamental for establishing and maintaining the structure and function of neural circuits. The axon plays a vital role in carrying cargoes to and from synapses. Proteins and organelles synthesized in the cell soma need to be transported through axons to reach the presynaptic terminal to support neuronal function. Correspondingly, signals received at distal axons need to be transduced and conveyed to the soma. These processes are essential for neuronal survival, differentiation, and maintenance. In that axonal transport in some neurons must be conducted through distances more than 1000 times the diameter of the cell body, the possibility is readily envisioned that even small deficits could markedly impact neuronal and circuit function.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors, is present in many brain regions, including hippocampus, cerebral cortex, and basal forebrain. BDNF plays a crucial role in cognition and memory formation by supporting the survival, differentiation, and function of neurons that participate in cognitive circuits. BDNF binds to its receptor, the tyrosine kinase TrkB, at the axon terminal where it activates TrkB-mediated signaling pathways including the mitogen activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK), phosphatidylinositol-3-kinase (PI3K) and phospholipase C-gamma (PLCγ). The proteins that participate in these signaling pathways are packaged onto endocytic vesicular structures to form the BDNF/TrkB signaling endosome[1-6] that are then retrogradely transported to the neuronal soma.

The microfluidic culture chamber is a very useful platform for studying axonal biology under normal conditions as well as in the setting of injury and disease[7,8]. By isolating axons from the cell bodies, the device has allowed one to study transport specifically in axons[8-10]. The PDMS based microfluidic platforms with 450 µm microgroove barriers used in this study were commercially purchased (see materials and equipment's table). To examine BDNF transport, we developed a novel technology to produce monobiotinylated BDNF (mBtBDNF). We took advantage of the biotin acceptor peptide, AP (also known as AviTag). It is a 15-amino acid sequence that contains a lysine residue that can be specifically ligated to a biotin by the *Escherichia coli* enzyme biotin ligase, BirA. We fused the AviTag to the C-terminus of the mouse pre-proBDNF cDNA by PCR (Fig 1A). The construct was cloned into the mammalian expression vector, pcDNA3.1 myc-his vector. We also cloned the bacterial BirA DNA into the pcDNA3.1 myc-his vector. The two plasmids were transiently co-transfected into HEK293FT cells to express both proteins. BirA catalyzed the ligation of biotin specifically to the lysine reside within the AviTag at the C-terminus of BDNF at a 1:1 ratio to produce monobiotinylated BDNF monomer. Biotinylated, mature BDNF with a molecular mass of ~18 kDa was recovered and purified from the media using Ni-resin (Fig 1C). The biotinylation of BDNF was complete, as judged by the inability to detect unmodified BDNF by immunoblotting (Fig 1D). Streptavidin conjugated quantum dots, QD 655, were used to label mBtBDNF to make QD-BDNF. The presence of the AviTag did not interfere with activity of BDNF as the mBtBDNF was able to activate phosphorylated TrkB (Fig 1E) and stimulate neurite outgrowth (Fig 1F) to the extent of recombinant human BDNF (rhBDNF). Immunostaining shown that QD-BDNF colocalized with TrkB in hippocampal axons, indicating that QD-BDNF is bioactive (Fig 1G). To study the BDNF transport, QD-BDNF was added to distal axon compartment of microfluidic cultures containing rat E18 hippocampal neurons (Fig 2A). QD-BDNF retrograde transport within axons was captured by real-time live imaging of the red fluorescent tag (supporting video S1, S2). By analyzing the kymograph generated, QD-BDNF was observed to be transported retrogradely at a moving velocity of around 1.06 µm/s (Fig 3A). GFP or mCherry-tagged BDNF have been used to track axonal movement of BDNF. The major drawbacks are that they are not bright enough for single molecule studies. Also, the presence of both anterograde and retrograde BDNF movements makes it difficult to evaluate whether or not the retrogradely transported BDNF was in a neurotrophin/receptor complex.

In this video, we demonstrate the techniques used to examine live trafficking of QD-BDNF in microfluidic chambers using primary neurons. The ultra-brightness and excellent photostability of quantum dots makes it possible to perform long-term tracking of BDNF transport. These techniques can be exploited to enhance studies of axonal function in AD, HD, and other neurodegenerative disorders.

PROTOCOL:

Surgical and animal procedures are carried out strictly according to the NIH Guide for the Care and Use of Laboratory Animals. All experiments involving the use of animals are approved by UCSD Institutional Animal Care and Use Committee

1. Plasmid cloning, expression and purification of mono-biotinylated BDNF (mBtBDNF)

Construct pre-proBDNFavi and BirA cDNA into pcDNA3.1 vector and co-express in HEK293FT cells[10]. Purify mBtBDNF using Ni-NTA beads according to previously published method of producing mature and biologically active mono-biotinylated nerve growth factor (mBtNGF)[10].

2. Preparation of microfluidic chambers

Microfluidic neuron culturing device makes it possible to fluidically isolate axons from neuron cell bodies. Assemble chambers with freshly coated coverslips right before each dissection. Microfluidic chambers used in this protocol are commercially purchased (see materials and equipment's table). Handwash and reused commercially purchased chambers up to 5 or 6 times.

2.1) Hand wash microfluidic chambers in 1% alconox. Rinse three times with Milli-Q water for 30 min each. Hand wash chambers again in 70% ethanol.

2.2) Lay out chambers to dry on parafilm in the laminar flow hood. Radiate and sterilize both sides of the chambers for 20 min under UV. Store sterilized chambers in a sterile 15 cm dish sealed with parafilm at room temperature.

2.3) Place 24x40 mm No. 1 glass coverslips in a glass container. Soak the coverslips in 35% hydrochloride acid overnight on a rotator. On the following day, rinse the coverslips three times for 30 min each with water.

2.4) Sterilize the coverslips one by one by dipping each coverslip into 100% ethanol and flaming over a Bunsen burner. Store dry coverslips in a sterile petri dish and keep it at room temperature until coating.

2.5) Lay out the coverslips in a 15 cm culture dish. Coat each coverslip with 0.7 ml of 0.01% poly-L-Lysine (PLL) and incubate in the hood at room temperature.

2.6) After an hour, rinse the coverslips three times with sterile water. Dry coverslips with vacuum and place each coverslip into a 6 cm culture dish.

2.7) To assemble the microfluidic chamber, place the chamber with microgroove side at the bottom onto the PLL coated coverslip with caution not to touch the microgrooves. Gently pressed down the chamber with a pipette tip to ensure the chamber was tightly sealed.

3. Dissection of neuronal culture and plate on chambers

3.1) Place two E17-E18 rat hippocampi (one brain) dissected in a 15 ml conical tube containing 2 ml dissection buffer (HBSS, no calcium, no magnesium, with 1% Pen/strep and 10 mM HEPES. Rinse the tissues three times with 5 ml dissection buffer each time. Remove the dissection buffer as much as possible and add 900 µl of fresh dissection buffer.

3.2) To digest the tissue, add 100 µl of 10x trypsin (2.5%) into the dissection buffer to make 1x working concentration. Place the conical tube in a 37°C water bath. After 10 min of digestion, add 100 µl of 10 mg/ml DNase I to a final concentration around 1 mg/ml.

3.3) Using a fire-polished Pasteur glass pipette, gently triturate the tissues by pipetting up and down 5-10 times. Right after trituration, quench the trypsin with 2 ml plating medium (Neurobasal with 10% FBS, 2 mM GlutaMax, 2% B27).

3.4) Leave the sample in the hood for 5 min to allow any debris of the tissues to settle down to the bottom. Carefully remove 2 ml of supernatant into a clean sterile 15 ml conical tube and centrifuge at 200 x g for 5 min to pellet the cells. Resuspend the pellet in 50 µl plating media

3.5) Count the cells with hemocytometer. Load 15-20 µl cell suspension (~40,000 cells) into one compartment of the microfluidic chamber. Place the chamber in the incubator for 10 min to allow the cells to attach to the coverslip. After 10 min, add more plating media to fill up both compartments of the chamber.

3.6) On the second day of dissection, completely replace the plating media with maintenance media (Neurobasal with 2 mM GlutaMax, 2% B27) in both the cell body and the axon compartment. Axons from the hippocampal neurons start to cross the microgrooves in day 3 and reach the axon compartment between day 5-7. During this period of time, replace half of the culturing media with fresh maintenance media every 24 ~ 48 h.

4. Axonal transport of QD-BDNF

4.1) Prior to live imaging of QD-BDNF axonal transport, deplete BDNF from both the cell body and axon compartments of the microfluidic chamber by thoroughly rinse both compartments with BDNF-free, serum free Neurobasal media every 30 min for 2 h.

4.2) During BDNF depletion, prepare the QD-BDNF conjugates. Mix 50 nM of mono-biotinylated BDNF dimer with 50 nM QD655-streptavidin conjugates in neurobasal media and incubate on ice for 60 min.

4.3) Remove media in the axon compartment and add 300 µl QD-BDNF with a final concentration of 0.25 nM for 4 h at 37^{o}C. To minimize the diffusing of QD-BDNF into the cell body compartment, it is very important to always maintain a higher level of media in the cell body compartment than in the axon compartment. Wash off unbound QD-BDNF after incubation before live imaging.

4.4) Carry out live imaging of QD-BDNF transport using an inverted microscope equipped with a 100x oil objective lens. Warm up the scope and the environmental chamber attached to it to a constant temperature (37^{o}C) and CO_2 (5%). Use a set of Texas red excitation/emission cubes to visualize the QD655 signal.

4.5) Acquire and capture time-lapse images within the middle axons at the speed of 1 frame/s for a total of 2 min using a CCD camera. Use microgrooves with no axons that have no QD and hence no signal as a control for infiltration.

4.6) Analyze BDNF transport using any image analysis software or NIH ImageJ.

REPRESENTATIVE RESULTS:

Production and purification of biologically active mono-biotinylated BDNF

The expression vector of BDNF fused with an AviTag sequence (GGGLNDIFEAQKIEWHE) was created according to a previously published protocol[10]. The molecular mass of the full-length fusion protein was predicted to be ~32 kDa (http://ca.expasy.org/tools/pi_tool.html). Monobiotinylated mature BDNF with a predicted molecular mass of 18 kDa (Fig 1A) was produced in HEK293FT cells and purified from conditioned media as described[10].

Different fractions were collected and separated on a 15% SDS-PAGE. Using a rabbit anti-Avi tag antibody, a band with a molecular weight of ~18 kDa was detected in the first fraction (Figure 1B). To test the purity of the preparation, samples were separated on SDS-PAGE and were stained by silver-staining. As shown in Fig 1C, the 18 kDa band was the predominant species in the first elution. Streptavidin-agarose beads pulldown assays were performed to assess the extent of biotinylation of the mBtBDNF preparation. Following pulldown with streptavidin-agarose beads, proteins that remained in the supernatant were precipitated with 7% trichloroacetic acid (TCA). These samples were analyzed by SDS-PAGE and the blots were probed with either anti-Avi antibodies (top) to detect the total protein or HRP-streptavidin to detect biotinylated proteins. Our results shown that all the signals were associated with the beads while no band was detected in the supernatant (Fig 1D). We thus conclude that mBtBDNF was biotinylated at an efficiency >99.9%.

To test the biological activity of mBtBDNF in binding and activating the TrkB receptor, we treated a 3T3 cell line that expresses TrkB with either rhBDNF (20 ng/ml) or purified mBtBDNF (20 ng/ml) for 10 min. Cells that received no treatment were also collected as control. Lysates were separated on a 4-12% SDS-PAGE and a rabbit anti-pTrkB antibody was used to probe the phosphorylated TrkB. As shown in Fig 1E, similar to rhBDNF, mBtBDNF was able to induce the phosphorylation of TrkB at a similar level. We also treated rat hippocampal neurons with either rhBDNF (20 ng/ml) or purified mBtBDNF (20 ng/ml) for 48 h. Purified mBtBDNF was able to stimulate hippocampal neurite outgrowth to the extent of rhBDNF (Fig 1F). To examine the co-localization of QD-BDNF with TrkB receptor, QD-BDNF was added to rat hippocampal axon for 4 h at 37^{o}C. Neurons were then fixed and immunostained for TrkB. As shown in Fig 1G, QD-BDNF co-localized with TrkB. We thus conclude that mBtBDNF is fully active in its biological function.

Live imaging of axonal transport of QD-BDNF in hippocampal neurons

Primary embryonic rat hippocampal neurons (E17-18) were dissected and plated into the cell body compartment of microfluidic chamber. At day 4 or 5, axons extended across the microgrooves into the axon compartment. At day 7, most axons grew into the axon compartment. QD-BDNF was made by incubating the streptavidin QD655 with mBtBDNF at a 1:1 ratio (QD:BDNF dimer) on ice for 1 h. QD-BDNF (0.25 nM) was then added to the axon compartment and incubated for 4 h at 37^{o}C prior to imaging (Fig 2A).

Following incubation, QD-BDNF was found to bind specifically to axons in the axon compartment. In the cell body compartment, QD signals were accumulated in most of the proximal axons and cell bodies indicating the QD-BDNF was internalized at the axons and

retrogradely transported to the cell bodies. Time-lapse image series of QD-BDNF signals within axons were carried out in the microgrooves. QD signals were observed in most of the microgrooves with axons while no QD signal can be seen in the microgrooves in the absent of axons, indicating little or no diffusion of QD signal from the axon compartment to the cell body compartment (Fig 2B).

In Fig 2C, an 80 µm long segment of axons of hippocampal neurons were fluorescently recorded for 100 s. Total of 6 QD-BDNF signals were clearly seen during the video recording. Three QD signals recorded moved unidirectionally towards the cell body (left side). The QD event labeled by white arrow (t:31 sec to t:101 sec) moved smoothly to the cell body crossing the entire field in ~ 70 s. Another QD event labeled by green arrow (t:1 sec to t:61 sec) moved retrogradely first. At t:21 sec, the QD-BDNF moved anterogradely for 10 to 20 s, and then changed direction again towards the cell body. The QD event labeled by yellow arrow (t:1 sec to t:71 sec) also moved retrogradely, but paused for ~20 s during the movement. QD-BDNFs that were not moving during this 100 s were considered stationary. Only moving objects were later analyzed to calculate moving velocity, average velocity, and time paused.

Data analysis in QD-BDNF transport kymograph

Kymographs were created from each movie recorded using Metamorph software. As shown in the representative kymographs of QD-BDNF transport (Fig 3A), QD-BDNF movements were recorded during 120 s in an 80 µm long segment of axons. In representative kymograph 1, QD-BDNF moved fast and smoothly towards left (cell body), crossed the field (80 µm) in ~ 60 s, with very brief pauses (supporting video S1). While in representative kymograph 2, a QD-BDNF traveled ~50 µm in 120 s retrogradely, with at least three segments of longer pauses (indicated by arrows) (supporting video S2).

Fig 3B is the scatter plot of moving velocity, average velocity, and paused time of each single QD-BDNF. The moving speed of the QD-BDNF was relatively fast, ranging from 0.47 ~ 1.97 µm/s with the mean of 1.06 ± 0.05 µm/s. The average velocity of QD-BDNF was calculated as travelling distance/time used. And because BDNF paused during its transport, average velocity was much lower than moving velocity with mean of 0.48 ± 0.03 µm/s (ranging from 0.17 ~ 1.59 µm/s). Paused time was also analyzed as a characterization of the BDNF movement. The mean duration of pauses was 15.88 ± 1.30 s (ranging from 6 ~ 33 s) (Table 1).

Figure Legends:

Figure 1: Purification of biological active, monobiotinylated BDNF (mBtBDNF). (A) A schematic drawing shows the production of mBtBDNF. Both pre-proBDNFavi and BirA were cloned into the pcDNA3.1myc-his vector as described[10]. (B) Different fractions of elutes from Ni-resin were collected and separated on a 15% SDS-PAGE gel. A rabbit anti-Avi tag antibody was used in the blot. A band with an apparent molecular weight of ~18 kDa was recognized in the elute, consistent with the predicted molecular mass for the mature mBtBDNF protein. (C) A silver staining gel shows the 18 kDa mBtBDNF was the predominant species in the elution fraction. (D) Purified mBtBDNF was incubated with streptavidin-agarose beads. The beads were washed and boiled in SDS loading buffer while the

supernatant was precipitated with TCA prior to SDS-PAGE analysis. The blot was probed with either anti-Avi antibody or with HRP-streptavidin. (E) 20 ng/ml of purified mBtBDNF or rhBDNF was added to a 3T3 cell line that expresses TrkB for 10 min. Lysates were harvested and separated on a 4-12% SDS-PAGE. Control cell lysate was also analyzed. The blot was probed with a rabbit anti-pTrkB antibody. Total TrkB was revealed using a mouse anti-TrkB antibody. (F) 20 ng/ml of purified mBtBDNF or rhBDNF was added to rat hippocampal neurons for 48 h. DIC images were taken to analyze neurite outgrowth. (G) QD-BDNF was incubated with rat hippocampal neuron axons for 4 h (scale bar 5 µm). Neurons were then fixed and immunostained for TrkB.

Figure 2: Single molecule imaging of retrograde transport of QD-BDNF in axons. (A) Top view: A schematic drawing of microfluidic chamber with primary neurons plated in the cell body compartment. Axons grew across the microgrooves into the axon compartment. QD655 labeled BDNF was added to the axon compartment. Side view: The media level in the axon compartment was kept lower than the cell body compartment to minimize diffusion of QD signal. (B) Red fluorescence images and DIC images of cell body compartment, microgrooves and axon compartment. QD-BDNF bound specifically to axons, internalized, and retrogradely transported to the cell bodies along the axons. No QD signal was observed in the microgrooves absent of axons. (C) Time-lapse video image series of QD-BDNF transport along the axon. Both moving and stationary QD-BDNF signals were present in most of the movies. At time t:1 sec, at least 4 QDs can be seen. After 10 s, two QDs (indicated by green and yellow arrows) are moving towards the cell body (left of the image). These two QDs move and pause in the first couple images and eventually move out of the images to the left (at ~ 70 s). Another QD moved into the field at t:31 sec (indicated by white arrow) and moved quickly without much pauses to the left.

Figure 3: QD-BDNF transport kymograph and data analysis. (A) Representative kymographs of QD-BDNF transport in primary hippocampal neurons. In kymo 1, a bright QD moved across the field at relatively fast speed with only a few short pauses. In kymo 2, the moving speed of the QDs was slower, and the movement was interrupted with more frequent and longer pauses. (B) Scatter plot of QD-BDNF moving velocity, average velocity (calculated as travelling distance/time used) and paused time. The mean moving speed of QD-BDNF was 1.06 ± 0.05 µm/s (ranging from 0.47 ~ 1.97 µm/s). The average velocity of QD-BDNF was 0.48 ± 0.03 µm/s (ranging from 0.17 ~ 1.59 µm/s). The mean duration of pauses was 15.88 ± 1.30 s (ranging from 6 ~ 33 s).

Video S1: Representative QD-BDNF transport video 1. Several QD-BDNF move fast towards left side (cell body) with very few short pauses.

Video S2: Representative QD-BDNF transport video 2. Several QD-BDNF move relatively slow towards left side (cell body) with frequent and long pauses.

Table 1: Data analysis of QD-BDNF transport.

DISCUSSION:

In this study, we report the development of a novel technique to produce biologically active, monobiotinylated BDNF (mBtBDNF) that can be used to trace axonal transport of BDNF. By

conjugating the protein to quantum dot streptavidin, and using a microfluidic chamber, the method allows one to detect axonal transport of BDNF in primary neurons with single molecule sensitivity, in real-time and with spatial and temporal resolutions. The tools used herein provide a means by which to study the molecular machines that mediate axonal transport of BDNF/TrkB signaling endosomes in neurons in health and disease.

BDNF-GFP, or –mCherry have been used to track axonal movement of BDNF in various neuronal cultures in vitro. These reagents offer one option for examining anterograde transport and, possibly, for examining autocrine activation of TrkB. However, there are major drawbacks for studying retrograde transport, the most salient of which is that GFP or mCherry are not bright enough for single molecule studies. Prior studies showed that under physiological concentrations of NGF, a single NGF dimer was internalized[11] and retrogradely transported[8]. Importantly, the use of QD BDNF also allows one to define the subcellular location at which BDNF binds its TrkB receptors. In using GFP or mCherry-tagged proteins, somal expression will result in the presence within the axon of proteins undergoing anterograde and well as retrograde transport. As such, it may be difficult to assess whether or not or where retrogradely transported BDNF encountered its receptor prior to retrograde transport. Indeed, as a speculation, retrograde transport within axons of BDNF-GFP or BDNF-mCherry produced in the same neuron may differ from that following binding of BDNF in the target of innervation.

Although chemical crosslinking has been used for producing biologically active nerve growth factor (NGF), the method introduced herein is superior. First, it is difficult to control precisely the extent of biotinylation; for example, each NGF was labeled with 5-9 biotin moieties[8,9,12]. Secondly, crosslinking can inactivate the protein. In the case of NGF, crosslinking necessitated the attachment to carboxyl groups[13]. In the case of BDNF, activity is often lost following chemical crosslinking to biotin. Finally, the extent of crosslinking may be incomplete. A recent report showed that ~0.8 biotin/BDNF molecule was produced by chemical crosslinking; as such ~20% of BDNF was not labeled[9]. The presence of unlabeled BDNF could complicate the interpretation of results.

Our technique offers the unique advantages that BDNF molecules are all labeled with a single biotin at the same site, thus constituting a homogeneous preparation of biotinylated BDNF. By conjugating to nano-fluorescent particles such as quantum dots, mBtBDNF can be used for tracking the pathways and processes that BDNF is internalized, trafficked within neurons: in dendrites, in axons as well as in cell bodies. This method makes possible the use of alternative tags for BDNF. The development of other types of nanoparticles promises additional benefits.

Defects in axonal trafficking and signaling of BDNF have been implicated in neurodegenerative diseases. Strong evidence has linked defective transport of BDNF to cortical-striatal atrophy in Huntington's disease[14,15]. Mutant Huntingtin protein interferes with the interaction between Huntingtin-associated protein-1 (HAP1) and dynein motor in sympathetic neurons, inhibits BDNF transport, and results in loss of neurotrophic support and neuronal toxicity[16-18]. Our techniques of tracking axonal movement of BDNF can serve as a valuable tool for studying axonal function in neurodegenerative disorders.

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DISCLOSURES:
No conflicts of interest declared.

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1

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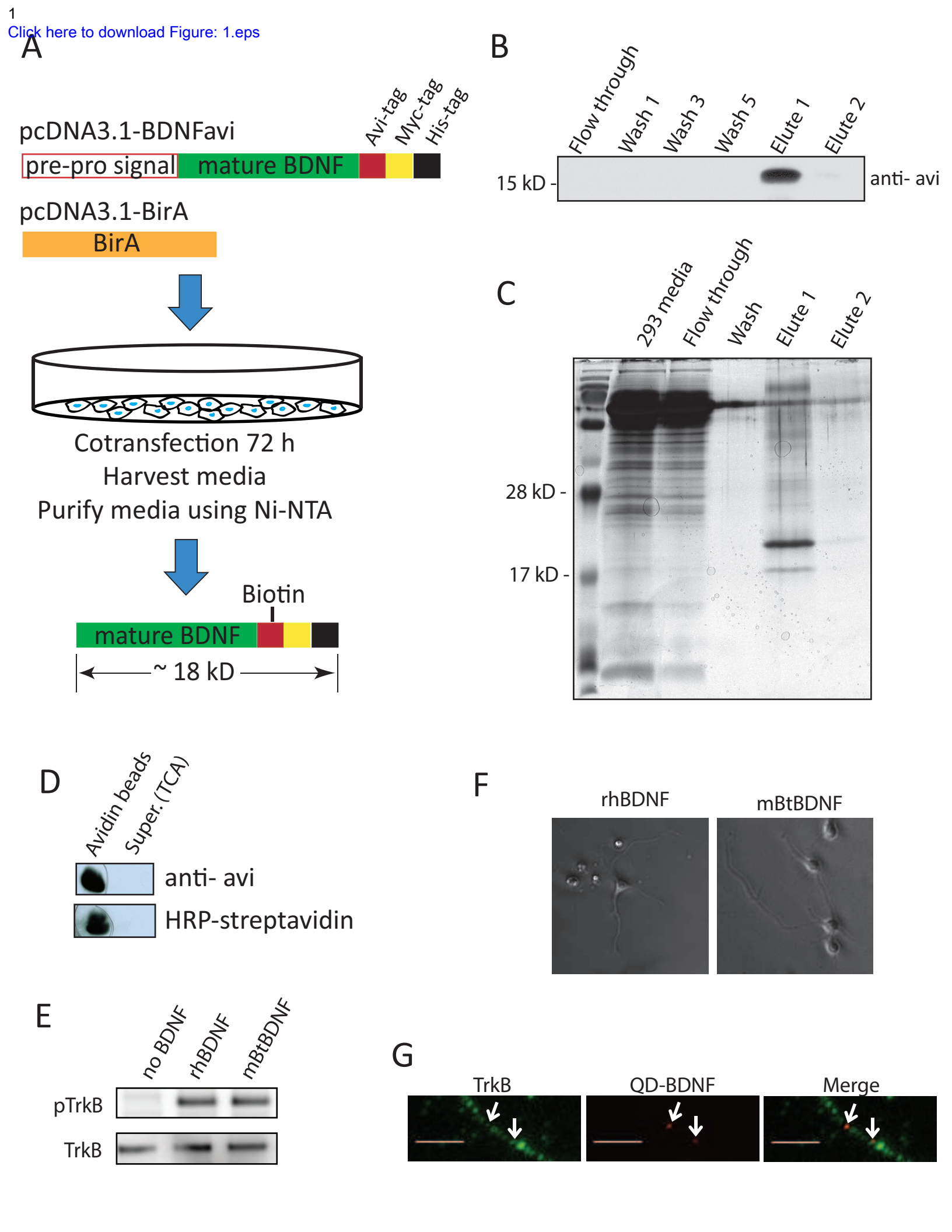

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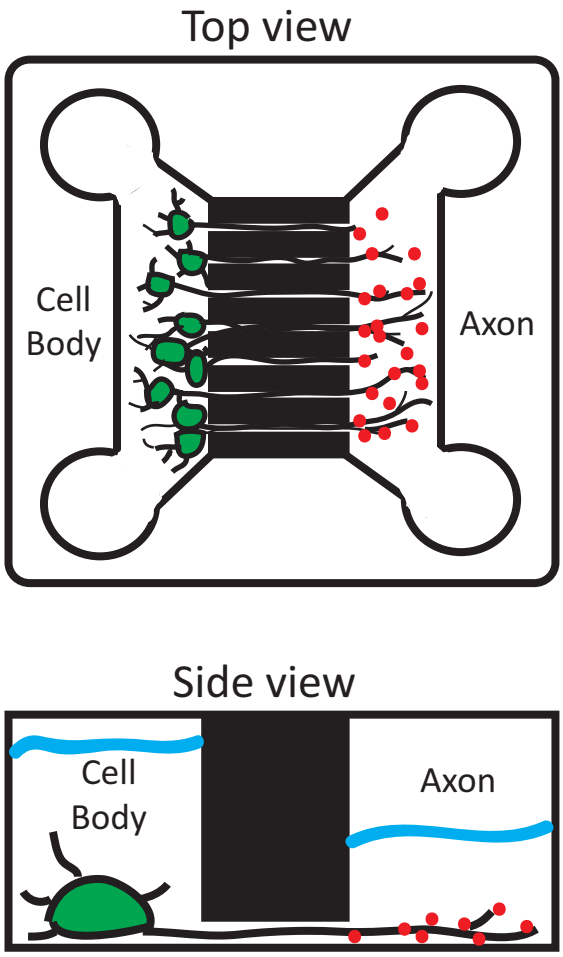

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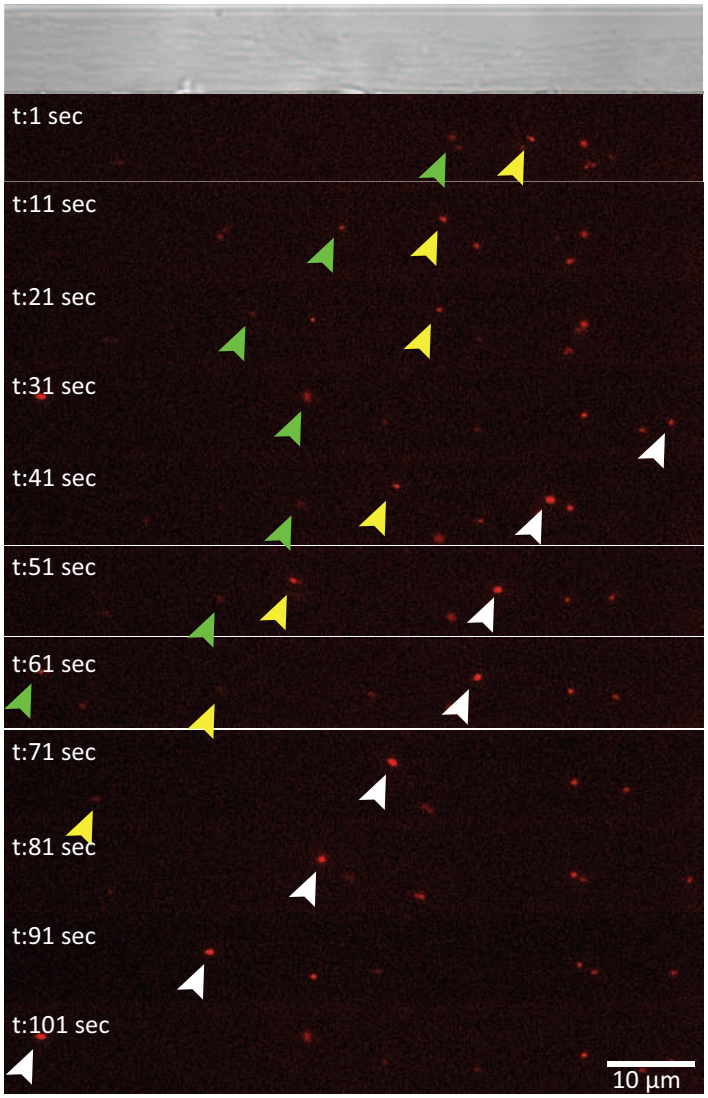

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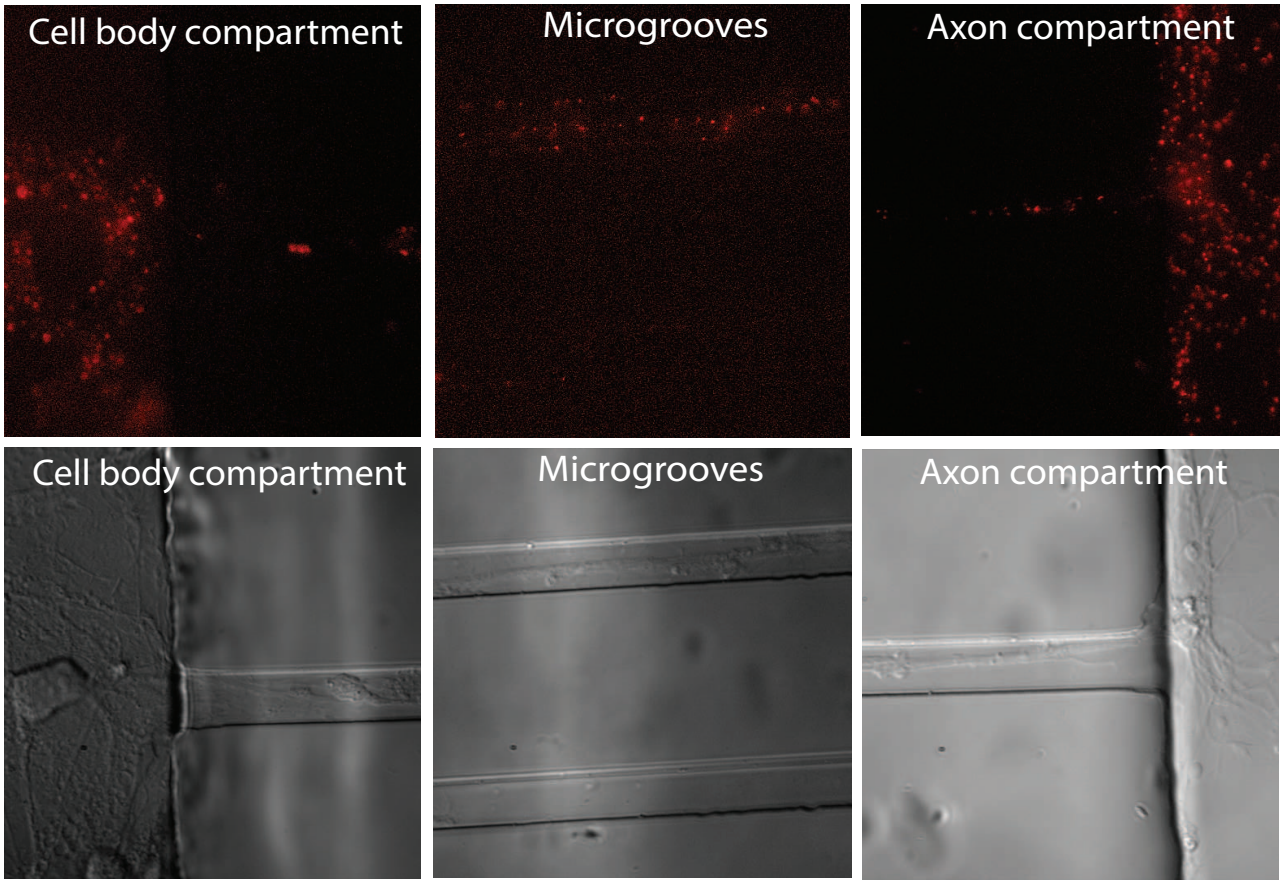

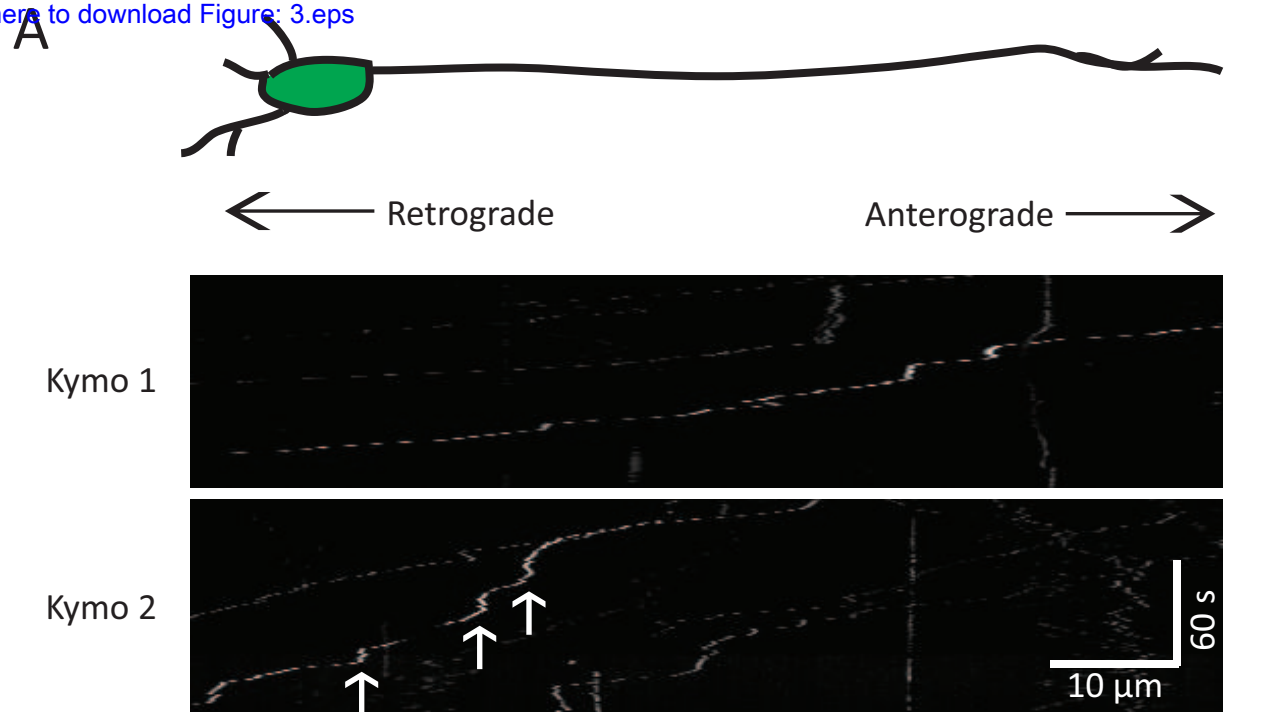

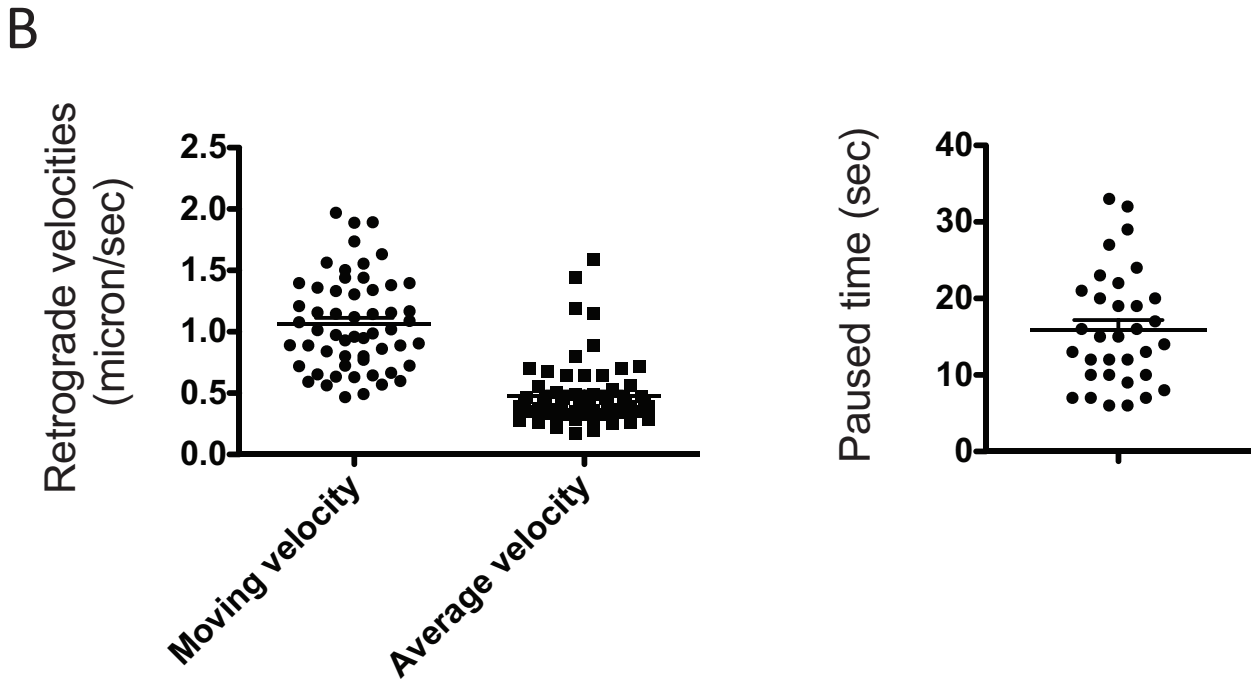

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	Minimum	Maximum	Mean	Std. error
Moving velocity (micron/sec)	0.47	1.97	1.06	0.05
Average velocity (micron/sec)	0.17	1.59	0.48	0.03
Paused time (sec)	6	33	15.88	1.30

Excel Spreadsheet- Table of Materials/Equipment

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Name	Company	Catalog Number
Platinum pfx DNA polymerase	Invitrogen	11708021
EcoRI	Fermentas	FD0274
BamHI	Fermentas	FD0054
HEK293FT cells	Invitrogen	R70007
DMEM-high glucose media	Mediatech	10-013-CV
d-biotin	Sigma	B4639
TurboFect	Fermentas	R0531
PMSF	Sigma	P7626
aprotinin	Sigma	A6279
Ni-NTA resins	Qiagen	30250
protease inhibitors cocktail	Sigma	S8820
silver staining kit	G-Biosciences	786-30
human recombinant BDNF	Genentech	
Microfluidic chambers	Xona	SND450
24x40 mm No. 1 glass coverslips	VWR	48393-060
poly-L-Lysine	Cultrex	3438-100-01
HBSS	Gibco	14185-052
DNase I	Roche	10104159001
Trypsin	Gibco	15090-046
Neurobasal	Gibco	21103-049
FBS	Invitrogen	16000-044
GlutaMax	Invitrogen	35050-061
B27	Gibco	17504-044
QD655-streptavidin conjugates	Invitrogen	Q10121MP
anti-Avi tag antibody	GenScript	A00674
streptavidin-agarose beads	Life Technology	SA100-04
trichloroacetic acid	Sigma	T6399
HRP-streptavidin	Thermo Scientific	N100
anti-pTrkB antibody	a generous gift from Dr M. Chao of NYU	
anti-TrkB antibody	BD Science	610101

1

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Author(s): Xiaobei Zhao, Yue Zhou, Matthew L. Pearn, William C Mobley, Chengbiao Wu

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List of changes
Please address the following editorial comments:

1) The microfluidic chamber seems to be an important part of the experiment, but there is no reference to directions on how to make/purchase the device other than in the materials list. It is okay to not include device fabrication (as it is not the point of this article), but the device should at least be briefly introduced.

Briefly addressed where the chambers were purchased at L98 in the introduction.

2) There are a few minor grammar/tense issues in the protocol:

Fixed the grammar/tense issues.

a) Steps 1 and 2 are followed by statements which could easily be written in the imperative form.

Rewrote steps 1 and 2 in the imperative form.

b) Step 3.6 contains the sentence "Axons from the hippocampal neurons started to cross the microgrooves in day 3 and reached the axon compartment between day 5-7." which should be put in the right tense.

Fixed.

c) Step 2.4 should start with the word "Sterilize" not "Sterilized".

Fixed.

3) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Fixed spelling and grammar issues.

4) Often reviewers request the addition of a large amount of details or explanations (For example reviewer # 3). We realize that, especially in the protocol section, brevity and clarity are important for a JoVE publication and expect the focus to be on providing a framework for the method presented rather than a comprehensive review of the research field. Please address each

comment in your rebuttal and note if you choose not to include the requested information in the text and the reasoning behind this decision.

We have added Fig 1F and Fig 1G to address reviewer's question. Instead of explaining all the experimental detail in the protocol, we talked about Fig 1 in the introduction and in the figure legends.

5) We do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript reports a new way to add biotin moiety to BDNF so that it can be labeled with Q-dot. The method will be useful to many in the field who is interested in understanding the role of BDNF in axonal trafficking. The method is clearly presented with beautiful results.

Major Concerns:

THe authors might consider bringing up the issues with GFP or RFP labeled BDNF in the introduction to emphasize the advantage of the current method. As it is, it is unclear until Discussion what is novel in their work.

The drawbacks of using GFP or mCherry-tagged BDNF have been inserted the introduction as suggested.

Minor Concerns:

There are many grammatical errors in the manuscript and typos.

Grammatical errors and typos have been fixed.

Reviewer #2:
Manuscript Summary:

The manuscript reports a novel method for imaging BDNF retrograde transport in cultured hippocampal neurons using quantum dot. The method allows a single-molecule resolution. The manuscript is well written.

Major Concerns:
None

Minor Concerns:

1. Need a space between number and unit. For example, 2ml (line 162) should be written as "2 ml". There are many of this kind of typos in the manuscript. The authors should go through the whole manuscript carefully and fix them.

Fixed.

2. "by handling only the sides of the chamber" (line 157) should be better described, because the top and bottom of the chamber are also sides.

Rewrote the sentence to “with caution not to touch the microgrooves. ”

3. Fig 1A (line 223) should be Fig. 1A. Figure 1B (line 228) should be Fig. 1B. Check the whole manuscript for consistency of figure citation.

All have been changed to as Fig 1A for consistency.

4. (line 229) "As in Fig. 1C" misses "shown".

It has been fixed

5. (line 228 - line 290) All clauses or sentences should use past tense.

Edited several sentences to past tense.

Reviewer #3:
Manuscript Summary:
In this manuscript, the authors describe a protocol for labeling BDNF with quantum dots in order to track the trafficking of signaling endosomes over long distances. This will be a very useful technique for the field and the video of how conjugation, purifications, elutions, etc… are performed will be particularly useful.

Major Concerns:

As the authors describe, such conjugation has been problematic in the past due to loss of bioactivity. The one criticism that I have is that the authors could take one more step to demonstrate bioactivity beyond phosphorylation in heterologous cells. There is precedent for different neurotrophins activating a TRK receptor similarly but their ability to influence survival is different. As such I worry that despite similar activation there is an outside chance that biotinylation of BDNF would change its function. I would like to see, a functional assay like long distance survival or if that doesn't work, a BDNF dose response using axon outgrowth as a read out. Either assay should be fairly straightforward

We performed neurite outgrowth experiment with mBtBDNF using recombinant human BDNF (rhBDNF) as a control. 20 ng/ml mBtBDNF or rhBDNF was added to rat hippocampal neuron culture for 48 h and cells were imaged to analyze neurite outgrowth. mBtBDNF was able to stimulate neurite outgrowth to the extent of rhBDNF. Fig 1F has been added to the manuscript.

Minor Concerns:

What is the purpose of the washing step of the microfluidic device—are the authors reusing them? I don't think this is recommended (should be commented on).

Chambers can be washed and reused up to 5 or 6 times. Comment added to the protocol step 2.

The authors control for this by examining infiltration of qdots into grooves with no axons (Should be mentioned in the text that this is a necessary control for every experiment).

This has been addressed in the protocol step 4.5.

Reviewer #4:

Manuscript Summary:

In this manuscript, Zhao and colleagues describe a method to follow imaging of single BDNF molecules in endosomes. This work is of interest and the methods are appropriately described.

Major Concerns:

Are there anterograde movements detectable ? By looking at the videos, it seems that some Qdots are going also anterogradely.

There were some anterograde movements but majority of the movements were retrograde.

Are the Qdots co-localizing with TrkB receptors ?

In rat hippocampal axons, Qdot signals co-localized with TrkB receptors using immunostaining. Fig 1G has been added to the manuscript.

Lane 212, the speed of acquisition is very low making fast movements of high speed vesicles not detectable via this technique. Authors should increase the frequency of acquisition.

Labeling BDNF with QD does allow faster acquisition, and we have tried higher speed acquisition in the past (10 frames/sec). For fast axonal transport events such as axonal transport of BDNF, the average moving speed is between 1-2 μm/sec, we have determined 1 frame/sec is sufficient to catch all movements of BDNF for our intended purposes.

Lane 253, it is very surprising that the authors wait so long before starting the acquisition time. TrkB receptor activation and endocytosis is a very fast event and retrograde movements are likely to be detectable within minutes after adding the ligand.

Consistent with other report (Xie et al., 2012), BDNF movements within axons can be observed 30-40 min after addition of QD-BDNF. The reason that we have chosen to image at 2-3 hrs is that at 2-3 hrs after adding the ligand, the movements of BDNF reaches steady state, allowing a more accurate measurement of parameters such as moving speeds, flux related to axonal transport .

Minor Concerns:

Lane 91, typo

Fixed.

Lane 100, was the 1 :1 ratio verified using the Pierce Biotin quantitation kit ?

The Avi tag also call AP tag was first described by Alice Ting of MIT. The lysine residue within the tag can only ligated to one biotin by E. coli biotin ligase (BirA). Therefore, unlike chemical crosslinking methods, the technique allows site-directed biotinylation. When we first adapted the technique for producing mBtNGF (Sung et al., 2011), we tested and confirmed that BirA did not ligate biotin to NGF without the Avi tag. So in theory, one BDNF or NGF dimer that has two biotin moieties/dimer can bind to two QDs when excess QDs are present. Based on the results of our pulldown experiment with streptavidin agarose, non-biotinylated BDNF or NGF was not detectable by immunoblotting, suggesting that we have achieved significantly high efficiency of biotinylation. We mixed mBtBDNF with QD strictly following 1:1 ratio (mBtBDNF or mBtNGF dimer:QD). The population of mBtBDNF or mBtNGF with two QDs will be a very rare event. We further demonstrated that when applied to axons of DRG neurons, all the QD-NGF signals within axons displayed an “on” and “off” characteristics, due to the intrinsic “blinking” property of a single QD, which is consistent with the 1:1 ratio of NGF dimer:QD (Sung et al., 2011). We produced mBtBDNF in exactly the same manner and we are confident that the 1:1 ratio holds true as well.

Lane 101, authors claim there is no interference of the AviTag on BDNF activity, however, the monobiotynilated BDNF but not the AviTag is tested in figure 1E.

The Avi tag was fused to the C-terminus of BDNF and the “K” residue within the Avi tag was specifically ligated to biotin at a 1:1 ratio, thus generating the BDNF-Avi-Biotin moeity. For simplicity, we call it monobiotinylated BDNF. We found that monobiotinylated BDNF (BDNF-Avi-Biotin) is capable of stimulating phosphorylation of TrkB similar to BDNF itself. We thus conclude the presence of the Avi tag does not interfer with BDNF activity.

Lane 146, can you specify 200 proof ethanol

Changed to 100% ethanol.

Lane 236, again quantification should be done using the Pierce kit.

Please see our response to Lane 100

Lane 352, this is overstated regarding the temporal resolution. Indeed, many papers have reported velocities within this range.

“that are orders of magnitude greater than with earlier methods” was deleted.

Lane 363, the term locus usually refers to genetic locus and should be modified.

Changed to “subcellular location”.

Lane 375-380, Xie et al 2012 and Liot et al 2013 have verified that crosslinking of biotin to BDNF does not modify BDNF activity.

In Xie et al 2012 figure 2A and figure 2C, purified biotin-BDNF or QD-BDNF induced phosphorylation of TrkB and phosphorylation of Erk are much lower than that induced from unmodified BDNF. In Liot 2013 figure 5E, the authors performed immunostaining of pCREB to show that biotin-BDNF was biologically active but they did not examine pTrkB and there was no quantitation of pCREB in their assay. They did not compare biotin-BDNF with non-biotinylated BDNF. In addition, although they did show that their biotin-BDNF preparation can be recognized by streptavidin-HRP by blotting, since biotin-BDNF can not be effectively separated from non-biotinylated BDNF on SDS-PAGE, the possibility of the presence of non-biotinylated BDNF in their preparation cannot be ruled out by this method. They did not perform any tests to measure the efficiency of biotinylation (Fig X).

Also, in Xie et al, 2012, there is no unlabeled BDNF as molecular species are separated by size exclusion chromatography. Text should be modified accordingly.

In Xie et al 2012, they mentioned “On average, 0.8 biotins were added onto each BDNF molecule” in the experimental part of preparation of biotin-BDNF. We have performed pulldown assays with stretavidin beads and collected the supernatant, we then precipitated all proteins in the supernantant with TCA. Our result demonstrates that non-biotinylated BDNF was not detectable in the supernantant, indicating significant efficiency of biotinylation of BDNF in our preparation.
