Video Article

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/sec. This system can be used to investigate mechanisms of disrupted axonal function in AD or HD, as well as other degenerative disorders.

Video Link

The video component of this article can be found at https://www.jove.com/video/51899/

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 1,000 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 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 (**Figure 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 (**Figure 1C**). The biotinylation of BDNF was complete, as judged by the inability to detect unmodified BDNF by immunoblotting (**Figure 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 (**Figure 1E**) and stimulate neurite outgrowth (**Figure 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 (**Figure 1G**). To study the BDNF transport, QD-BDNF was added to distal axon compartment of microfluidic cultures containing rat E18 hippocampal neurons (**Figure 2A**). QD-BDNF retrograde transport within axons was captured by real-time live imaging of the red fluorescent tag (**Supporting videos S1, S2**). By analyzing the kymograph generated, QD-BDNF was observed to be transported retrogradely at a moving velocity of around 1.06 μm/sec (**Figure 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 ultrabrightness 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)

NOTE: Construct pre-proBDNFavi and BirA cDNA into pcDNA3.1 vector and coexpress in HEK293FT cells[10]. Purify mBtBDNF using Ni-NTA beads according to previously published method of producing mature and biologically active monobiotinylated 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 5-6x.

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. 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.
3. Place 24 x 40 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.
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.
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.
6. After 1 hr, rinse the coverslips three times with sterile water. Dry coverslips with vacuum and place each coverslip into a 6 cm culture dish.
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

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 3x with 5 ml dissection buffer each time. Remove the dissection buffer as much as possible and add 900 μl of fresh dissection buffer.
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. Using a fire-polished Pasteur glass pipette, gently triturate the tissues by pipetting up and down 5-10x. Right after trituration, quench the trypsin with 2 ml plating medium (Neurobasal with 10% FBS, 2 mM GlutaMax, 2% B27).
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
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.
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 hr.

4. Axonal Transport of QD-BDNF

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 hr.
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.
3. Remove media in the axon compartment and add 300 µl QD-BDNF with a final concentration of 0.25 nM for 4 hr at 37 °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. 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 °C) and CO_2 (5%). Use a set of Texas red excitation/emission cubes to visualize the QD655 signal.
5. Acquire and capture time-lapse images within the middle axons at the speed of 1 frame/sec 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.
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 (**Figure 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 **Figure 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 (**Figure 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 **Figure 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 hr. Purified mBtBDNF was able to stimulate hippocampal neurite outgrowth to the extent of rhBDNF (**Figure 1F**). To examine the co-localization of QD-BDNF with TrkB receptor, QD-BDNF was added to rat hippocampal axon for 4 hr at 37 °C. Neurons were then fixed and immunostained for TrkB. As shown in **Figure 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 hr. QD-BDNF (0.25 nM) was then added to the axon compartment and incubated for 4 hr at 37 °C prior to imaging (**Figure 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 (**Figure 2B**).

In **Figure 2C**, an 80 µm long segment of axons of hippocampal neurons were fluorescently recorded for 100 sec. 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 sec. 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 sec, 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 sec during the movement. QD-BDNFs that were not moving during this 100 sec 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 sec 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 sec, with very brief pauses (**Supporting Video**

S1). While in representative kymograph 2, a QD-BDNF traveled ~50 µm in 120 sec retrogradely, with at least three segments of longer pauses (indicated by arrows) (**Supporting Video S2**).

Figure 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/sec with the mean of 1.06 ± 0.05 µm/sec. 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/sec (ranging from 0.17-1.59 µm/sec). Paused time was also analyzed as a characterization of the BDNF movement. The mean duration of pauses was 15.88 ± 1.30 sec (ranging from 6-33 sec) (**Table 1**).
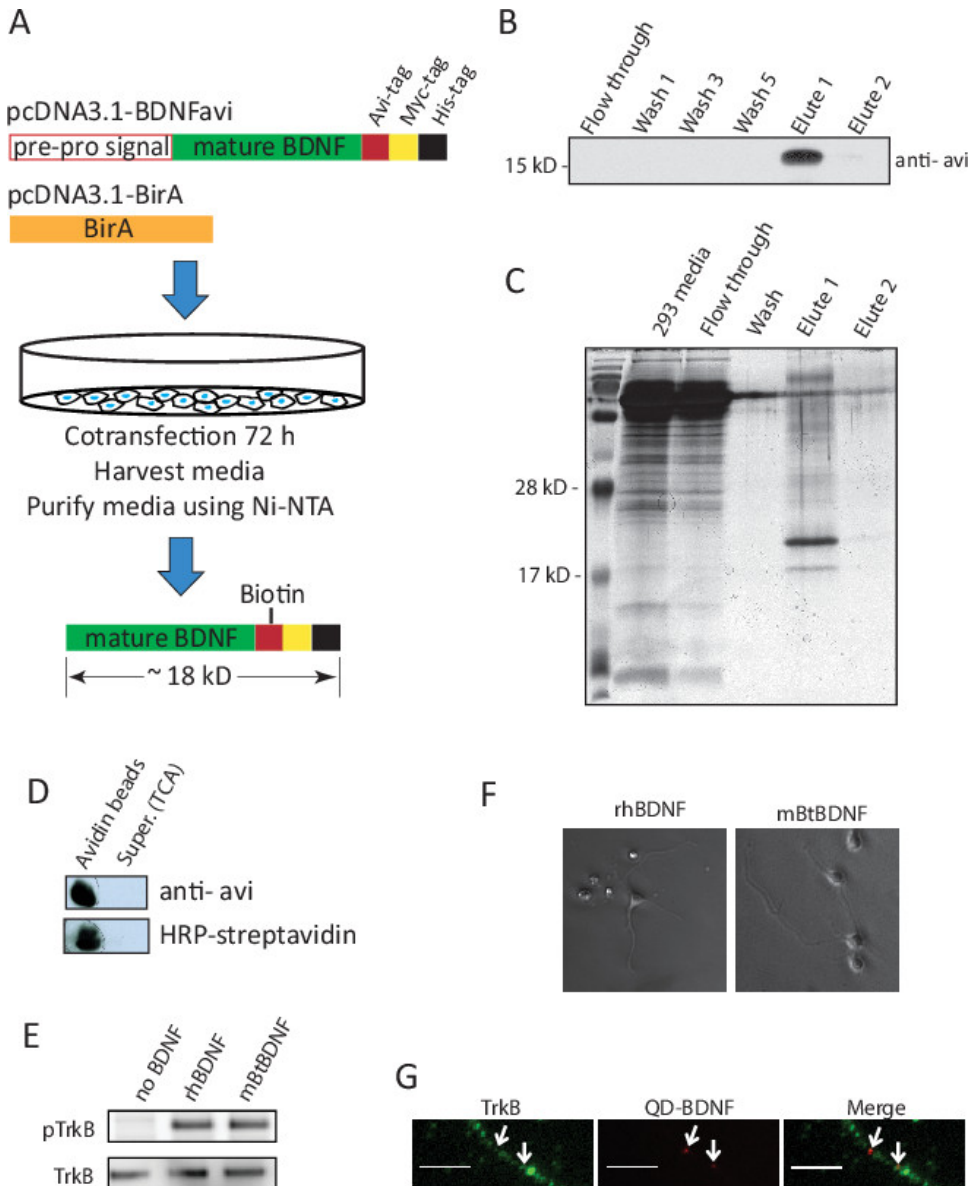

Figure 1. Purification of biological active, monobiotinylated BDNF (mBtBDNF). (**A**) A schematic drawing shows the production of mBtBDNF. Both pre-proBDNFavi and BirA were cloned into the pcDNA3.1myc-his vector as described[10]. (**B**) Different fractions of elutes from Ni-resin were collected and 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 hr. DIC images were taken to analyze neurite outgrowth. (**G**) QD-BDNF was incubated with rat hippocampal neuron axons for 4 hr (scale bar 5 µm). Neurons were then fixed and immunostained for TrkB.
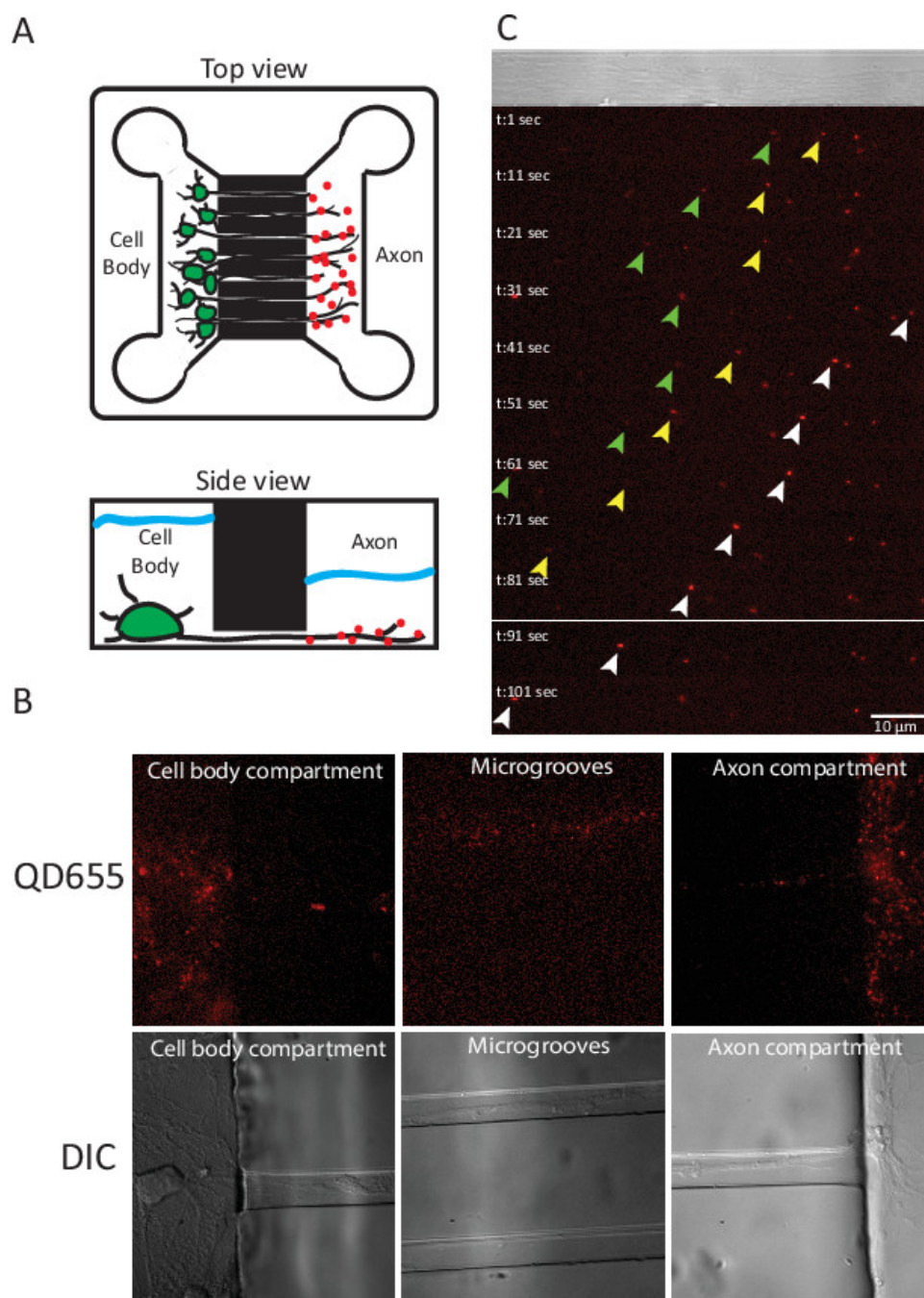
Figure 2. Single molecule imaging of retrograde transport of QD-BDNF in axons. (**A**) Top view: A schematic drawing of microfluidic chamber with primary neurons plated in the cell body compartment. Axons grew across the microgrooves into the axon compartment. QD655 labeled BDNF was added to the axon 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 sec, 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 sec). Another QD moved into the field at t:31 sec (indicated by white arrow) and moved quickly without much pauses to the left.
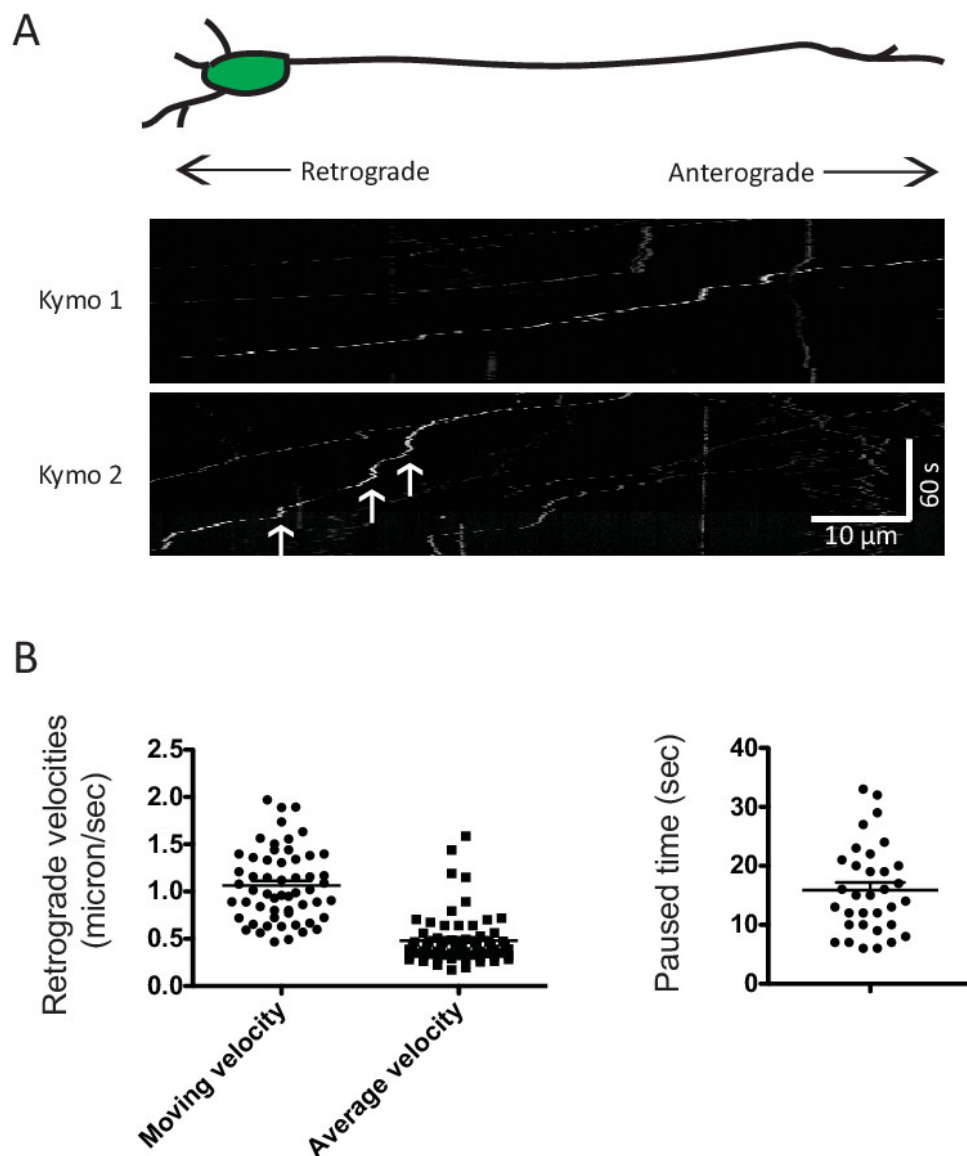
Figure 3. QD-BDNF transport kymograph and data analysis. (**A**) Representative kymographs of QD-BDNF transport in primary hippocampal neurons. In kymo 1, a bright QD moved across the field at relatively fast speed with only a few short pauses. In kymo 2, the moving speed of the QDs was slower, and the 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/sec (ranging from 0.47-1.97 µm/sec. The average velocity of QD-BDNF was 0.48 ± 0.03 µm/sec (ranging from 0.17-1.59 µm/sec). The mean duration of pauses was 15.88 ± 1.30 sec (ranging from 6-33 sec).

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

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

	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

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 nanofluorescent 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.

Disclosures

No conflicts of interest declared.

Acknowledgements

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