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 m/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.

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