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Abstract

The efficient recycling of synaptic vesicles (SVs) during neuronal activity is central for sustaining brain function. During intense neuronal activity, the dominant mechanism of SV retrieval is activity-dependent bulk endocytosis (ADBE). Here we describe a method to monitor ADBE in isolation from other SV endocytosis modes, via the uptake of large fluorescent fluid phase markers in primary neuronal culture. Furthermore, we outline how to monitor ADBE using this approach across a field of neurons or in individual neurons.

Keywords: Dextran, endocytosis, vesicle, neuron, presynapse.

1. Introduction

The localised endocytosis of synaptic vesicles (SVs) at the presynapse is essential to sustain neurotransmission in mammalian brain. Three modes of endocytosis maintain SV supply, which are triggered by specific patterns of neuronal activity. During very mild neuronal activity, ultrafast endocytosis (UFE) is the dominant SV endocytosis mode. UFE forms small endosomes directly from the plasma membrane, before a second SV generation step [1,2]. This pathway rapidly saturates during action potential trains, meaning it will likely provide a minor contribution to SV supply during physiological patterns of activity [3]. Action potential trains also trigger clathrin-mediated endocytosis (CME) [4], however this mode also saturates during periods of high neuronal activity [5]. During high neuronal activity a different endocytosis mode is triggered, called activity-dependent bulk endocytosis (ADBE). ADBE is a two-step process, with large invaginations forming bulk endosomes directly from the plasma membrane [5], with SVs budding from these endosomes thereafter [6].

The physiological role of ADBE in neuronal function is still undetermined, mainly because molecules specific to this process are only now starting to be identified [7]. Central to defining the role of key molecules in ADBE are assays that only report the triggering of this mode, rather than either UFE or

CME. Commonly used approaches to monitor endocytosis with either fluorescent dyes such as FM1-43 [8] or genetically-encoded reporters such as synapto-pHluorins [9] are not appropriate, since they report the retrieval of membrane or proteins that are retrieved by all three endocytosis modes. One assay that monitors ADBE specifically is the internalisation of large fluid-phase markers such as dextrans. High molecular weight versions of these molecules (40 kDa and 70 kDa) have difficulty entering single SVs formed via CME, meaning they almost exclusively report ADBE (Fig. 1) [10]. The extent of dextran labelling of UFE will be minimal, since this endocytosis mode provides a very minor contribution to SV retrieval during high neuronal activity [3]. Therefore high molecular weight dextran molecules provide a simple optical estimation of the number of nerve terminals performing ADBE.

This assay can be used for individual neurons or populations of neurons [11-13,7]. A population-based approach is usually used when all neurons in primary culture are treated in a similar manner, for example after application of a drug. It is also used to compare between cultures derived from animals with different genotypes, or after viral transduction. The single neuron approach is typically used after transient transfection with either shRNA or overexpression plasmids, since the low transfection efficiency of this delivery method negates a population-based response.

2. Materials

2.1 Equipment

1. Sealed imaging chamber with embedded platinum wires such as a Warner Imaging chamber (Warner RC-21BRFS).

2. Electrical field stimulator (*see* **Note 1**).

3. Perfusion system, for example a gravity flow system with peristaltic pump.

4. Inverted epifluorescence microscope with standard rhodamine filters (plus appropriate cyan fluorescent protein (CFP)/ green fluorescent protein (GFP) filters if required to identify individual transfected neurons) and a high numerical aperture (NA) X20 or X40 objective (*see* **Note 2**). A cooled charged-coupled device digital camera connected to the microscope plus the associated operating software will also be required.

5. Primary cerebellar granule neurons (or other primary neuronal cultures) cultured on coverslips compatible with the imaging chamber (*see* **Note 3**).

6. Fiji (https://imagej.net/Fiji) and Microsoft excel or other similar software to analyse the gathered images.

2.2 Solutions and reagents

1. Imaging buffer for cerebellar granule neurons: 170 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM TES (*N*-tris(hydroxyl-methyl)-methyl-2-aminoethane-sulfonic acid), 5 mM $NaHCO_3$, 5mM glucose, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, pH7.4 (*see* **Note 4**). (If using high potassium solution (*see* **Note 1 and 11**) instead of electrical stimulation, high potassium imaging buffer is as above but with the substitution of 50 mM KCl for 50 mM NaCl.)

2.Imaging buffer for cortical/hippocampal neurons (if required): 136 mM NaCl, 2.5 mM KCl, 2mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (2-[4-(2-hydrosxyethyl)piperazin-1-yl]ethanesulfonic acid), pH 7.4 supplemented with 10 µM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and 50 µM APV (DL—2-amino-5-phosphopentanoic acid).

3. Dextran solution: 2 mM of 40 kDa TMR (tetramethylrhodamine)-dextran: in water (40 X stock) stored at -20 °C until use (*see* **Note 5**).

4. Vacuum grease.

3. Methods

3.1 Field TMR-dextran uptake

All steps are performed at room temperature (*see* **Note 6**).

1. Take a coverslip with primary neurons attached and replace the culture medium with the appropriate imaging buffer. Allow the cells to equilibrate for a minimum of 10 min (*see* **Note 7**).

2. Dilute the TMR-dextran in imaging buffer to 50 µM ready for use. You will only need sufficient diluted TMR-dextran solution to fill the imaging chamber – approximately 230 µl.

3. Assemble the imaging chamber (*see* **Note 8**) but do not seal the chamber at the top using a coverslip or the clamp from the chamber platform. Set the stimulation settings on the stimulator (40 Hz, 10s is sufficient to activate ADBE [5]) and connect to the imaging chamber.

4. Add the TMR-dextran solution to the cells and stimulate immediately (*see* **Note 9**).

5. Immediately following termination of stimulation, aspirate the TMR-dextran solution (with either a pipette or vacuum pump) and wash the coverslip twice with imaging buffer (*see* **Note 10**).

6. Finish assembling the imaging chamber with the top coverslip and platform clamp and mount on the microscope. Connect to the perfusion apparatus and continuously wash the coverslip for 2-5 min with imaging buffer to ensure that all of the non-internalised TMR-dextran has been removed (*see* **Note 11** for an adapted version of the protocol using high potassium solution to stimulate internalisation of TMR-dextran).

7. Focus on the neurons using brightfield illumination and then take approximately 10 representative fields of as equal neuronal density as possible in both the brightfield (for future reference) and the rhodamine fluorescent channel for TMR-dextran uptake (*see* **Notes 12-14**).

8. If Z-stacks were taken to image the TMR-dextran, first combine those images using the Z-projection tool in Fiji to generate one image (*see* **Note 15**).

9. The images should then be thresholded to remove any background fluorescence that is not due to TMR-dextran. The remaining TMR-dextran puncta can be automatically counted using the Analyse Particles tool in Fiji (*see* **Note 16**). The fields from the same coverslip are then averaged to determine the overall TMR-dextran uptake from that coverslip (see Fig. 2 for example data generated with this method).

10. An identical process should be undertaken for unstimulated controls. This is employed to calculate the average background signal, which is subtracted from the evoked signal (*see* **Note 17**).

3.2 Individual neuron TMR-dextran uptake

1. Perform the protocol above for field TMR-dextran uptake from steps 1 to 6, but using neurons that have been previously transfected with a marker and/or your plasmid of interest (*see* **Notes 18-19**).

2. Following mounting of the coverslip, locate a field with transfected neurons and take an image in both channels to record the position of the transfected neurites as well as the TMR-dextran uptake in the field. Repeat until multiple fields have been imaged (*see* **Note 20**).

3. As with the field TMR-dextran uptake, include non-stimulated controls in the experiment design. These should be conducted in exactly the same manner with the same incubation time with the TMR-dextran solution as with the stimulated coverslips.

4. To analyse TMR-dextran uptake, first overlay the transfected neurite image on the TMR-dextran uptake image using Fiji (*see* **Note 21**). Manually mark the TMR-dextran puncta which overlay with the transfected neurite in the ROI manager and record the number of puncta per transfected neurite in the field.

5. Calculate TMR-dextran uptake per length of neurite by measuring the length of the neurite using a tool such as the Simple Neurite Tracer in Fiji (https://imagej.net/Simple_Neurite_Tracer) and dividing the TMR-dextran uptake for that field by this (see Fig. 3 for example data generated with this method).

6. Repeat the analysis for the non-stimulated controls and subtract the non-specific uptake to calculate the evoked TMR-dextran uptake.

4. Notes

1. Electrical field stimulation is the most physiological method to stimulate cells, however, it is possible to use a modified version of this protocol to use high KCl solution (*see* **Note 11**). This evokes a clamped depolarisation to initiate exocytosis and subsequent ADBE that is equivalent to a field stimulus of 80 Hz for 10 s [11,14].

2. An inverted microscope is required so that the oil objective can be in contact with the bottom of the sealed imaging chamber. A high NA objective is required to efficiently detect the dim TMR-dextran puncta. For single neurite experiments, a minimum X40 objective is recommended to ensure sufficient resolution to identify TMR-dextran uptake from individual neurites.

3. For example, if using a Warner Imaging chamber, coverslips smaller than 25 mm in diameter will not completely seal the imaging chamber leading to leakage and larger than 25 mm in diameter coverslips will not fit the platform into which the imaging chamber is subsequently placed.

4. A concentrated (ten times) stock of the imaging buffer can be prepared and stored at -20 °C. On the day an aliquot can be thawed, diluted and warmed to room temperature for imaging.

5. TMR-dextran is sensitive to both light and temperature. It is delivered as a lyophilised powder for immediate storage at -20 °C. Before reconstituting, allow to warm briefly at room temperature. It is recommended to put the appropriate volume of water into the packaging and gently reconstitute using a pipette set to approximately a fifth of the total volume (to ensure the TMR-dextran is

completely dissolved and no clumps remain). A failure to reconstitute completely will lead to large fluorescent aggregates being present during imaging. Once in solution, aliquot and store at -20 °C until required. Once an aliquot is thawed for use, it should be kept on ice in the dark until dilution into imaging buffer for incubation with the cells.

6. The experiment could be conducted at 37 °C, although the loading of the TMR-dextran would have to be performed inside a temperature-controlled chamber.

7. The equilibration step at the start of the experiment is essential if using cerebellar granule neurons, since these neurons are grown in depolarising media and require to be repolarised before the experiment.

8. To ensure a good electrical contact, at the start of each imaging day the platinum wires should be gently rubbed with some fine sandpaper to remove any salt deposit. If vacuum grease is required to assemble the imaging chamber, for example a Warner imaging chamber, the wires should also be cleaned carefully to ensure that they are free from grease. During assembly, vacuum grease should be applied with just sufficient to seal the coverslips. An excess of vacuum grease can squeeze into the chamber, potentially blocking the perfusion tubing exits on the chamber as well as preventing the platinum wires efficiently conducting the stimulation. Excess of vacuum grease in the chamber also provides a surface for non-specific binding of TMR-dextran which will introduce high background levels of fluorescence into the experiment.

9. It is important to also include non-stimulated coverslips in order to control for any non-specific binding or uptake from the neurons. The coverslip should be set up in exactly the same way as for a stimulated coverslip and the TMR-dextran incubated with the cells for the same period of time as the simulated cells, for example 10s to be equivalent to a 40 Hz 10 s stimulus. These coverslips can be used for background subtraction during the analysis step.

10. Directly aspirating the TMR-dextran solution from the coverslip is the most effective way to remove the majority of the non-internalised TMR-dextran quickly, ensuring uptake reflects stimulus evoked uptake.

11. Adapted protocol using high potassium solution instead of electrical stimulation: dilute the TMR-dextran in the high KCl imaging buffer instead of the NaCl imaging buffer and use this solution in place of the field stimulation. Wash in the same manner as for the field stimulation protocol and assemble the imaging chamber.

12. The output of the field TMR-dextran uptake assay is reliant on the density of the field of neurites, for example a dense field will have more neurites and thus likely more TMR-dextran uptake. Therefore it is imperative to image fields of as equal density as possible to ensure comparable results between coverslips and preparations. To control for this it is possible to pre-incubate cultures with fluorescent-tagged antibodies that recognise lumenal epitopes of SV proteins, such as synaptotagmin-1 [15]. This will provide an estimation of the density of nerve terminals per field, which can be used to normalise the TMR-dextran signal.

13. It can be beneficial to take a small Z-stack of images in the TMR-dextran channel, either manually or automatically, to capture the entire TMR-dextran uptake in a field that may be in different planes of focus. These images can then be combined together at the analysis stage.

14. During the course of the image acquisition, the background can become increasingly bright and diffuse. This is almost certainly due to clumps of TMR-dextran that had adhered to the coverslip or TMR-dextran solution that had adhered to the edges of the chamber, leaching fluorescence into the field of view. This can be minimised by periodically switching the perfusion on and re-washing the coverslip or by imaging with continuous perfusion. The latter option will use a large volume of buffer however.

15. The TMR-dextran assay is binary in terms of its readout. Therefore it reports whether or not a given nerve terminal has internalised TMR-dextran or not. It is not possible to extract information on the extent of TMR-dextran uptake per nerve terminal (e.g. the amount of fluorescence intensity). Thus, using a maximum fluorescence Z-projection algorithm to compress a Z-stack of images increases the ability of a macro to accurately identify the TMR-dextran puncta without compromising the image data because it minimises the background fluorescence and maximises the TMR-dextran signal.

16. A macro to automatically process the images requires thresholding of the individual images and then count the particles of the correct size that represent a nerve terminal (approximately 1- 3.5 um^2). These values will depend on the microscope and the magnification of the objective and should be optimised by the user. This is important to ensure the accuracy of the counting process. If the range includes particles that are too small to be nerve terminals or too large representing particles that could not be internalised, the macro will over-estimate the count. Conversely, if the range is too stringent, the macro will under-estimate the number of nerve terminals accumulating TMR-dextran.

17. The background signal can vary between preparations of cells and batches of dextran but is typically 10 - 20 % of the evoked signal.

18. The density of the cultures is still important for analysis of TMR-dextran uptake in individual neurons because if the cultures are too dense, it is difficult to resolve TMR-dextran internalisation in the transfected neurite against a background of uptake in a field of untransfected neurites.

19. To minimise bleed through between the channels, when transfecting with a fluorescent marker, maximise the spectral difference between the rhodamine emission of the TMR-dextran and the label of interest, for example mCerulean is better than GFP. Far-red reporters (such as Alexa-647 or cypHer) are also compatible partners for TMR-dextran imaging [15].

20. The analysis for this method can be time consuming, therefore determine in advance the number of fields required for the analysis bearing in mind the values are combined to provide a coverslip average.

21. There are several ways to do this but overlaying the images using the Channels Tool in Fiji (Menu: Image → Colour → Channels Tool) or a plugin such as the Align RGB plugin (https://imagej.net/Align_RGB_planes) is effective because it allows toggling of the transfected neurite channel to check that the TMR-dextran puncta are located on the neurite in question. Using a method which permanently overlays the channels can make identification of the TMR-dextran more difficult depending on the fluorescence intensity of the transfection marker.

5. Figure legends

Fig. 1: *Overview of the tetramethylrhodamine (TMR)-dextran uptake assay to monitor activity-dependent bulk endocytosis (ADBE)*. Graphic shows 40 kDa TMR-dextran molecules in red which, following neuronal stimulation, are not accumulated into single retrieving synaptic vesicles but can be accumulated into larger endosomes formed by ADBE. This results in labelled endosomes which can then be detected by fluorescent imaging, selectively identifying nerve terminals which have undergone ADBE.

Fig. 2: *Example data using field tetramethylrhodamine (TMR)-dextran uptake assay.* Cerebellar granule neurons (CGNs) were stimulated at 40 Hz for 10 s in the presence of 50 µM TMR-dextran. The non-internalised TMR-dextran was immediately washed off and the resulting uptake imaged. (**A**) Representative images from CGNs either stimulated at 40 Hz for 10 s (left-hand panels) or mock stimulated (right hand panels). TMR-dextran uptake in nerve terminals is visualised as bright puncta (upper panels). Mock stimulated panels show background fluorescence likely from autofluorescence

and cellular debris which is generally dimmer and does not form discrete puncta. There can be a contribution from non-specific adherence of TMR-dextran. Taken together, background typically represents about 10-20 % of the evoked uptake. The lower panels illustrate the thresholded images used for the analysis. Note the difference in the size and shape of the thresholded areas – in the stimulated panel discrete lines of small puncta representative of TMR-dextran uptake along the neurites are visible, compared to larger aggregate areas present in the unstimulated panel. The range settings for the automated particle counter tool have to be set with respect to the individual microscope to allow accurate and appropriate quantification of the number of TMR-dextran positive nerve terminals. (**B**) Zoomed areas of panel A shown in the red boxes. (**C**) Quantification of the presented whole field thresholded stimulated image (Stim) and mock stimulated image (Mock) shown in (**A**) using the Analyse Particles tool in Fiji with the following settings: size 4-8 pixels and circularity 0-1. Images taken at a magnification of x20. Scale bar = 30 µm.

Figure 3: *Example data using the individual neuron tetramethylrhodamine (TMR)-dextran uptake assay*. Cerebellar granule neurons (CGNs) were transfected with an mCerulean (mCer) vector 72 hrs prior to incubation with 50 µM TMR-dextran solution and stimulation at 40 Hz for 10 s. Non-internalised TMR-dextran was washed away immediately before imaging the neurons for the presence of TMR-dextran containing nerve terminals. (**A**) Neurite transfected with mCer. (**B**) The same field, showing TMR-dextran uptake. (**C**) A merged image of the mCer image and the image showing TMR-dextran uptake. Arrows represent nerve terminals labelled with TMR-dextran, indicating they have undergone activity-dependent bulk endocytosis (ADBE). (**D**) The axon trace image generated using the Simple Neurite Tracer plugin in Fiji to quantify the length of neurite. Images taken at a magnification of x40. Scale bar = 5 µm.

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Figure 1

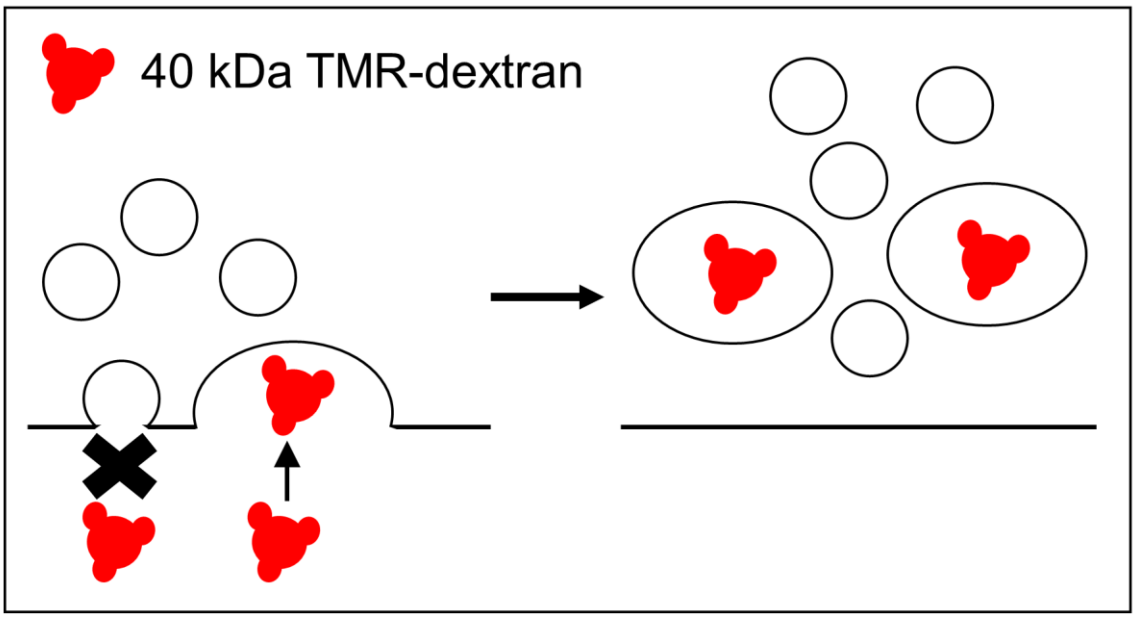

Figure 2

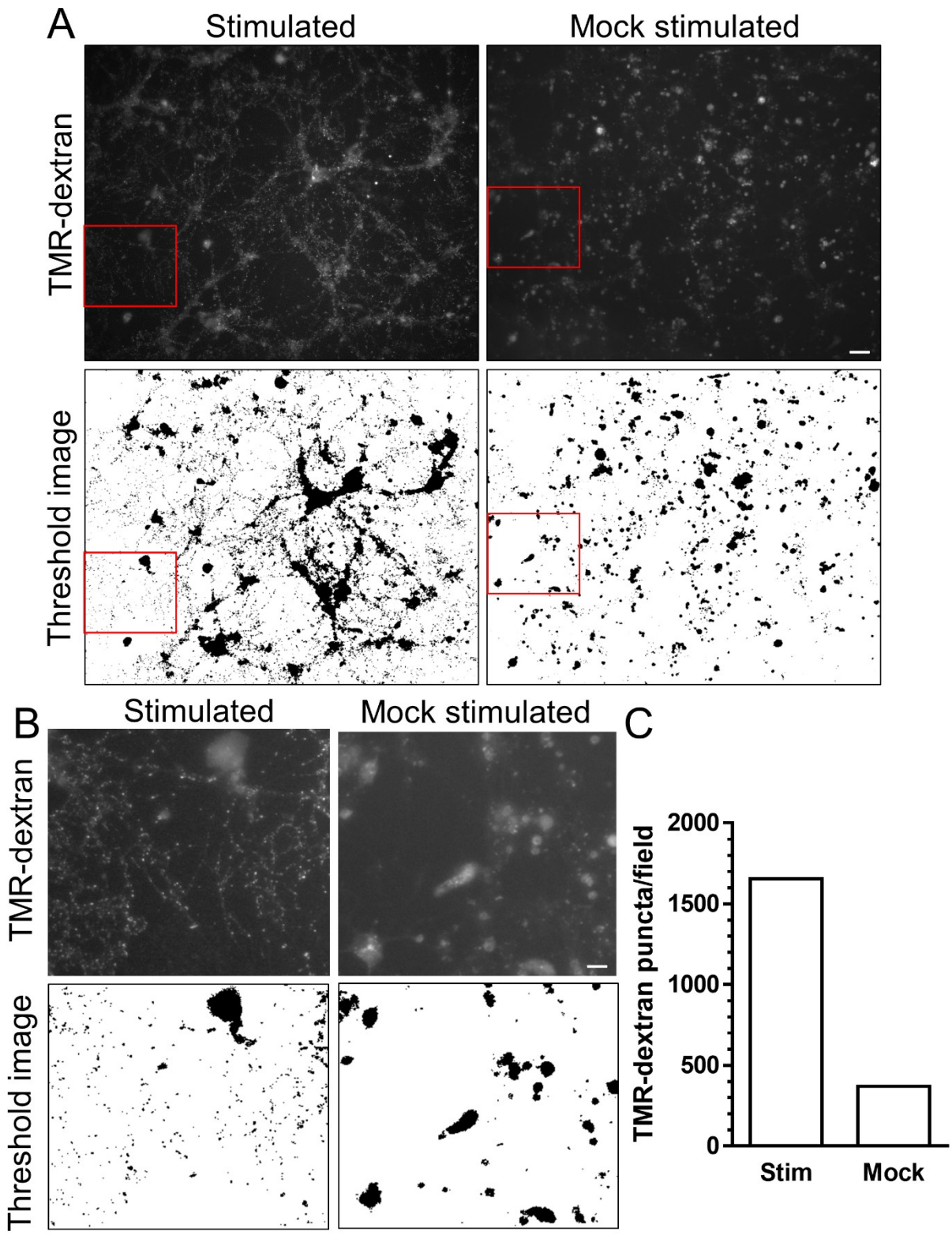
A
Stimulated
Mock stimulated
TMR-dextran
Threshold image
B
Stimulated
Mock stimulated
TMR-dextran
Threshold image
C
TMR-dextran puncta/field
2000
1500
1000
500
0
Stim
Mock

Figure 3