27 September 2013

Susan Rasakham, Ph.D.

Science Editor

JoVE

Dear Dr. Rasakham,

We thank the Reviewers for their useful comments and suggestions, all of which have been incorporated into the attached revised manuscript #51477R3. Below are our detailed responses to each of the Reviewers’ points.

Reviewer #1

Minor concern: We have added a brief discussion of image manipulation standards and pitfalls, including a reference.

Minor revisions: The grammatical and typographical errors have been fixed.

Reviewer #2

Minor comments:

1. We had intended Figure 3, which contains both low- and high-power views of a typical terminal, to serve the purpose of orienting the reader, similar to the reviewer’s suggestion of a cartoon (and in addition to the figure’s depiction of deconvolution). We have now made this feature of Figure 3 more explicit, including in the legend. The figure is also referred to in the text with reference to the anatomy and size of a typical nerve terminal, again in lieu of a cartoon (see response to Reviewer 3, Minor point 1 below).

2. Yes, the exocytosis of a macroendosome (ME) is relatively rare and if we catch the event, it is usually only one endosome. Out of many experiments we have at most perhaps ten 4D sequences in which two endosomes exocytose within the same few minutes. Also, as the reviewer infers, movement of MEs is not the norm; they usually remain static or exhibit slight Brownian motion.

3. We have clarified the method of getting a sequence of time points from multiple fixed preparations. We now make it clear that many more MEs remained on average after, say, 30 minutes of live imaging than after 30 minutes in the dark. Also clear is that we attribute the extra remaining MEs to phototoxic inhibition of their normal behavior, particularly exocytosis.

4. The Reviewer is correct—both examples in Figure 3 are intended primarily to show reduced blurriness with deconvolution. As mentioned in response to the Reviewer’s point 1 above, the reason we included two examples was to show that deconvolution works at both high and low magnification. Moreover, the low-power view serves to introduce the reader to the appearance of a typical entire nerve terminal as well as to serve as an example of low power deconvolution (see also response to Reviewer 3, point 8).

Reviewer # 3 (Major Points)

1. Reference to commercial products including software is allowed only in the table of materials; we have added additional software choices. Reference to axons has been removed.

2a. We have briefly discussed the issue of temperature in the text. In the experiments described, preparations were cooled mainly to delay bacterial contamination. Other experiments were performed at room temperature, however, with similar results. Experiments with mouse motor terminals (not discussed) were performed at 37 deg.

2b. In the experiments described, refrigerated saline (~4 deg initially) was used, and replaced at approximately 5 min intervals (between imaging sequences). The chamber was pre-chilled before use. Temperature control was not precise.

2c. The Reviewer is correct that adequate time before washout of FM dyes is required if all endocytosis is to be picked up. We studied this time course previously using tissue fixed at various times. Even as endocytosis continues, some MEs begin to dissipate into vesicles within seconds of internalization. In the present experiments, however, our goal was to record endosomal behavior as soon as possible after stimulation.

2d. Rinsing time has been clarified. We begin imaging 4-5 min after stimulation—three rapid ~1 min rinses, followed by refocusing.

2e. The appropriate electrical stimulation protocol has been added.

3a. Characteristics of the Zeiss Achroplan water-immersion objective used have been added to the Table.

3b. ImageJ is now mentioned in the Table. We were unable to test it due to problems with metadata associated with our Slidebook images. We have eliminated mention of “no neighbors” deconvolution because it is too specific to our particular application. It was chosen mainly to permit comparison of different image types. Other choices (e.g. constrained iterative) occasionally lost relevant information.

4. Issues regarding movement artifact, including sampling error, pointed out by the Reviewer are indeed relevant to the manuscript and are now discussed along the following lines. The time required per z-stack was typically 1-10 sec. It depended on the exposure time per z plane and the number of planes imaged. The exposure time and number of images chosen represent compromises among image quality, image noise, exposure time, depth of field and phototoxicity. Excitation light is shuttered off between exposures. During the total acquisition time (1-10 sec), the faster-moving endosomes in our preparation move around 100-1000 nm, based on our measurements of their velocity over longer times. We occasionally saw blurring consistent with movement. Most endosomes were stationary or moved very little. Sampling error is indeed a possibility generally. In our preparation, movement observed in early conventional time-lapse experiments (~1 sec frame rate) was consistent with that recorded in 4D at slower rates, indicating that the Nyquist criterion was met. Movement of the whole image field was slight in our experiments and always monotonic throughout a sequence of frames. The possibility of sampling error due to such movement is now briefly mentioned in the revised manuscript; again our suggestion would be to image rapidly in a single plane in order to quantify it—or simply view the field continuously.

5. We agree with the Reviewer’s point regarding the importance of phototoxicity as well as the difficulty in adequately quantifying it. Our newer camera was about 3X more sensitive than the older one (added to text); the absolute sensitivity of both can be found on the vendors’ websites. We have added an estimate of the rate of photobleaching with the fluorophores used.

6. We have added the (Sutter Instruments) light source and related equipment to the Table.

7. The Reviewer’s interpretation is correct. While somewhat improved by oversampling and deconvolution, z-resolution remains ~4X less than x-y resolution. We recently imaged the preparation described with a new state-of-the art Olympus confocal microscope being demonstrated at our institution, and were surprised to see very little improvement in z-resolution compared either to the microscope described here or to our older Zeiss LSM 510 confocal.

8. We have not “caught” endosome fusion, possibly because the fusion occurs before live imaging commences (after FM rinsing). The Reviewer’s suggestion (AE “kiss and run”) is a possibility. We consider it unlikely, however, based on previous experiments which we have now repeated in order to add more statistical certainty to the results. Putative ME-AE fusion (i.e., double-labeled structures) is relatively rare. If these structures were created by FM uptake during our typical 2 min depolarization, they should become less rare with longer exposure to FM. However, when preparations labeled with LysoTracker were incubated for 1 hr in FM1-43, none took up that dye to become double-labeled (n=2 experiments). We also have many experiments using sucrose (10-15 min) with bath-applied FM, and have seen no increase in double-labeled structures. It remains possible that depolarization is necessary to promote partial fusion of AEs to the plasma membrane. Unfortunately, if we extend the time of KCL depolarization we not only see no double-labeling but the LysoTracker staining fades, presumably due to loss or the AE proton gradient with generalized cellular damage. We also have no evidence from any source that AEs (putative lysosome-like organelles) fuse with the membrane at all, nor that if they did, they would take up an extracellular probe while not losing their own probe (LysoTracker). Thus we consider the Reviewer’s proposed mechanism unlikely but cannot yet rule it out. We are working on ways to either image sooner after stimulation or to promote, via some intervention, a larger number of putative ME-AE fusion events for study.

9. Deconvolution is indeed a well-established technique, but the other methodology of the paper is no more uncommon. We feel that an explicit illustration of deconvolution—the critical tool that allows good image quality without confocal optics--seems appropriate. While this could be associated with another figure, Figure 3 is intended to serve several other purposes as well (see also responses above to Reviewer 2, points 1 and 4). In particular, we now use this figure to point out that FM1-43 background staining (vesicle haze) indicates extent of the bouton’s intracellular space and therefore location of its plasma membrane (see response to Minor Concern 1 below). The figure is also the paper’s only example of a conventional stereo image (in this case an anaglyph), the most common representation of 3D and 4D data and perhaps the best to illustrate sharpening by deconvolution. It seems to us that it would overburden one of the other figures if it were constructed to make its own point (ME exocytosis or ME-AE fusion) plus all of the points made by Figure 3.

Reviewer #3 (Minor Concerns)

1. The “3D volume views” produced by our software comprise rotation about a single axis; we have added detail to Figure 1 and legend to make this clear. The FM vesicle haze indeed defines the location of plasma membrane. We determined this previously from EM serial section reconstructions. Vesicles in these reconstructions are congruent with FM haze seen at light level. The use of haze to define the bouton membrane is now briefly explained in the text, and illustrated in Figure 3. To elaborate, all of the bouton, when viewed at light level, is accessible to vesicles. Limiting structures (the postsynapse and Schwann cell cap) are extracellular with respect to the bouton. There is an internal “compartment” that is towards the back of the bouton (away from the postsynapse) and rich in mitochondria; vesicles are dispersed within this region but not excluded from it. There are also areas within the bouton that contain microtubules and to which some AEs seem to be confined, but there is no apparent compartment or membrane-bound structure here visible at either EM or light level.

2. The Reviewer is correct regarding our conclusions from the 2007 paper cited. At that time, experiments using preparations fixed at different time points indicated that endosomes vanished over time, while EM experiments provided direct evidence of their budding into 50 nm vesicles and occasionally splitting into smaller endosomes. We wondered what happened to plasma membrane proteins and extracellular fluid (particularly Ca++) after budding, but didn’t have a potential answer until we directly visualized exocytosis with 4D live imaging (Stewart et al, 2012). As stated, we now know that those MEs seen to exocytose clearly vanished by that mechanism, rather than by totally budding into vesicles. We still don’t know whether these MEs are “spent” (having budded away all vesicular proteins), are a separate class of MEs that exocytose without budding, or both.

3. We have unfused the references--thanks.