Journal of Visualized Experiments

Visualization of Endosome Dynamics in Living Nerve Terminals with Four-Dimensional Fluorescence Imaging

--Manuscript Draft--

Manuscript Number:	JoVE51477R3
Full Title:	Visualization of Endosome Dynamics in Living Nerve Terminals with Four-Dimensional Fluorescence Imaging
Article Type:	Methods Article - JoVE Produced Video
Keywords:	neuroscience, nerve, endosome, lysosome, deconvolution, 3D, 4D, epifluorescence, labeling
Manuscript Classifications:	5.1.370.350.515.458: Microscopy, Fluorescence; 7.4.299.482: Endocytosis; 8.1.158.610: Neurosciences
Corresponding Author:	Robert S Wilkinson, PhD washington university St. Louis, MO UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	WILK@wustl.edu
Corresponding Author's Institution:	washington university
Corresponding Author's Secondary Institution:	
First Author:	Richard S Stewart, PhD
First Author Secondary Information:	
Other Authors:	Richard S Stewart, PhD
	Ilona M Kiss
Order of Authors Secondary Information:	
Abstract:	Four-dimensional (4D) light imaging has been used to study behavior of small structures within motor nerve terminals of the thin Transversus abdominis muscle of the garter snake. Raw data comprises time-lapse sequences of 3D z-stacks. Each stack contains 4-20 images acquired with epifluorescence optics at focal planes separated by 400-1500 nm. Steps in the acquisition of image stacks, such as adjustment of focus, switching of excitation wavelengths, and operation of the digital camera, are automated as much as possible to maximize image rate and minimize tissue damage from light exposure. After acquisition, a set of image stacks is deconvolved to improve spatial resolution, converted to the desired 3D format, and used to create a 4D "movie" that is suitable for variety of computer-based analyses, depending upon the experimental data sought. One application is study of the dynamic behavior of two classes of endosomes found in nerve terminals—macroendosomes (MEs) and acidic endosomes (AEs)—whose sizes (200-800 nm for both types) are at or near the diffraction limit. Access to 3D information at each time point provides several advantages over conventional time-lapse imaging. In particular, size and velocity of movement of structures can be quantified over time without loss of sharp focus. Examples of data from 4D imaging reveal that MEs approach the plasma membrane and disappear, suggesting that they are exocytosed rather than simply moving vertically away from a single plane of focus. Also revealed is putative fusion of MEs and AEs, by visualization of overlap between the two dye-

	containing structures as viewed in each three orthogonal projections.
Author Comments:	Separate files containing screenshots for workflow will be supplied separately for videographer
Additional Information:	
Question	**Response**

Journal of Visualized Experiments

Cambridge, MA 02139

Dear Editor:

Attached is our manuscript, "Visualization of Endosome Dynamics in Living Nerve Terminals with Four-Dimensional Fluorescence Imaging," which we are submitting for publication in the Journal of Visualized Experiments. We believe that the JoVE's unique format is well-suited for presentation of our imaging set-up, particularly in order to illustrate use of focusing and other automated controls necessary for generation of 4D image data with adequate speed and precision. Moreover, editing, enhancement and other manipulations of video data sets are ideally presented in a mixed printed/video format.

Author Contributions: RSS: performed experiments, analyzed data; IML: analyzed data; RSW: analyzed data, prepared manuscript.

We have been corresponding with Jove Editor Zhao Chen and, in her absence, Leora Cramer.

Potential expert Reviewers:

Dr. Rita Balice-Gordon, University of Pennsylvania Medical School, rbaliceg@mail.med.upenn.edu

Dr. W. J. Betz, University of Colorado Medical School, Bill.Betz@ucdenver.edu

Dr. Gavriel David, University of Miami Medical School, G.David@miami.edu

Dr. Karl Magleby, University of Miami Medical School, KMagleby@miami.edu

Dr. Rodney Parsons, University of Vermont Medical School, Rodney.parsons@uvm.edu

Dr. Laurence Trussell, Oregon Hearing Research Center, trussell@ohsu.edu

Yours sincerely,

Richard S. Stewart

Ilona M. Kiss

Robert S. Wilkinson

Visualization of Endosome Dynamics in Living Nerve Terminals with Four-Dimensional Fluorescence Imaging

Richard S. Stewart, Ilona M. Kiss, Robert S. Wilkinson

Correspondence to: Robert S. Wilkinson at wilk@wustl.edu; (314) 362-2300

Keywords: neuroscience, nerve, endosome, lysosome, deconvolution, 3D, 4D, epifluorescence, labeling

Stewart, Richard S.
Dept. Cell Biology and Physiology
Washington University School of Medicine
Saint Louis, MO 63110, USA
rstewart@wustl.edu

Kiss, Ilona M.
Dept. Cell Biology and Physiology
Washington University School of Medicine
Saint Louis, MO 63110, USA
Ilona.m.kiss.15@dartmouth.edu

Wilkinson, Robert S.
Dept. Cell Biology and Physiology
Washington University School of Medicine
Saint Louis, MO 63110, USA
wilk@wustl.edu

Short abstract

Four-dimensional (4D) imaging is utilized to study the behavior and interactions among two types of endosomes in living vertebrate nerve terminals. Movement of these small structures is characterized in three dimensions, permitting confirmation of events such as endosome fusion and exocytosis.

Long abstract

Four-dimensional (4D) light imaging has been used to study behavior of small structures within motor nerve terminals of the thin Transversus abdominis muscle of the garter snake. Raw data comprises time-lapse sequences of 3D z-stacks. Each stack contains 4-20 images acquired with epifluorescence optics at focal planes separated by 400-1500 nm. Steps in the acquisition of image stacks, such as adjustment of focus, switching of excitation wavelengths, and operation of the digital camera, are automated as much as possible to maximize image rate and minimize tissue damage from light exposure. After acquisition, a set of image stacks is deconvolved to improve spatial resolution, converted to the desired 3D format, and used to create a 4D "movie" that is suitable for variety of computer-based analyses, depending upon the experimental data sought. One application is study of the dynamic behavior of two classes of endosomes found in nerve terminals—macroendosomes (MEs) and acidic endosomes (AEs)—whose sizes (200–800 nm for both types) are at or near the diffraction limit. Access to 3D information at each time point provides several advantages over conventional time-lapse imaging. In particular, size and velocity of movement of structures can be quantified over time without loss of sharp focus. Examples of data from 4D imaging reveal that MEs approach the plasma membrane and disappear, suggesting that they are exocytosed rather than simply moving vertically away from a single plane of focus. Also revealed is putative fusion of MEs and AEs, by visualization of overlap between the two dye-containing structures as viewed in each three orthogonal projections.

Introduction

Time-lapse imaging of living tissue provides visual access to dynamical structure-function relations that cannot be appreciated in fixed or living preparations imaged at a single point in time. Often, however, the tradeoff for access to temporal information is a decrease in optical resolution. High numerical aperture oil-immersion objectives are impractical in living tissue because of their narrow range of focus, leaving water immersion or dry objectives as the only alternatives. Moreover, the increased resolution afforded by confocal optics cannot be utilized in some living preparations due to phototoxicity from the relatively high levels of illumination required [1,2]. Lastly, while several real-time or time-lapse optical techniques are available that offer enhanced resolution, their applicability is limited to preparations where structures of interest can be positioned within a few hundred nanometers of the objective [1]. The method described makes use of relatively low-cost equipment, is versatile, yet offers improved resolution compared to more commonly-used time-lapse techniques. It is intended for use in individual laboratories as well as imaging facilities.

The method utilizes conventional epifluorescence microscopy, combined with a sensitive digital camera and with hardware designed to rapidly acquire sets of images at slightly different focal planes (z-stacks). Each z-stack is digitally deconvolved to increase resolution. One feature of 3D time-lapse (4D) imaging is precise tracking of moving organelles or other structures. When properly set up, imaged structures do not go out of focus, and movement in all three directions can be observed and quantified. Thus it is impossible for a stained structure to disappear over one or more time-lapse frames merely by drifting above or below a narrow focal plane. The method also serves as a sensitive tool for assessing the interactions and possible fusion of small structures. Conventional epifluorescence or confocal images of structures near the diffraction limit (a few hundred nm) do not confirm fusion even if merged images show overlap of their respective labels [3]. Fusion is suggested, but it remains possible that the objects are separated horizontally or vertically by a distance that is below the diffraction limit. Three- or four-dimensional imaging, in contrast, permits viewing the objects in each of three orthogonal directions. The appearance of fusion in all three views increases the level of certainty. And, in some living preparations, directed or Brownian movement of putatively fused objects provides further proof when both labels move together in time. Of course, when near the diffraction limit the level of certainty in discerning structures from background, or showing that they contain two dyes (fusion), is not absolute. If applicable, specialized techniques such as fluorescence resonance energy transfer (FRET) [4] are more appropriate.

Protocol

1. Stain the preparation with supravital dyes

1.1. For the garter snake dissection protocol see Stewart *et al.* 2012; Teng *et al.* 2007 [5,6]. Reptilian tissue remains physiological for longer times, and with less bacterial contamination, when kept at low temperatures (see below). Mammalian tissue is usually maintained at room temperature or higher.

1.2. For lysosomal vital dye staining, dissolve the dye in cold reptilian Ringers solution 1:5000 (0.2 μM). Incubate at 4 ^{o}C for 15 min. Wash several times with cold Ringers. Image as soon as possible.

1.3. For FM1-43 staining using KCl stimulation, dilute the dye in high-KCl Ringers 1:500 (7 μM). Incubate at 4 ^{o}C for 30-60 sec maximum. Wash quickly three times with cold Ringers, ~1 min per rinse. Image as soon as possible.

1.4. For FM1-43 staining using hypertonic (sucrose) stimulation, dilute the dye in 0.5 M sucrose Ringers as above. Incubate at 4 ^{o}C for 2 to 5 min. Wash as in 1.3 above with cold Ringers. Image as soon as possible.

1.5. For FM1-43 staining via electrical stimulation, see Teng *et al.* 2007 [6]. Briefly, the preparation is placed a dish containing physiological saline solution and the dye. The nerve is stimulated with a preprogrammed train of 200 μsec rectangular pulses (~7 V, 30 Hz, 18 sec). Wash as in 1.3 above with cold Ringers. Image as soon as possible.

2. Configure the preparation for imaging

2.1. Orient the preparation so that structures of interest are as close as possible to the microscope objective.

2.2. If an inverted microscope is used, utilize a chamber whose bottom contains a thin round cover slip. Typically, the imaging chamber should have a thin stainless steel bottom with a 25 mm diameter #1 thickness glass cover slip at the center. If an upright microscope is used, a bottom coverslip is not required but is useful to permit imaging with transmitted light (e.g., differential interference contrast, DIC) to orient the specimen and locate objects of interest.

2.3. Choose an appropriate objective to obtain the highest possible 3D resolution. There are tradeoffs among numerical aperture (n.a.), working distance, magnification, and type of lens (dry, water- and oil-immersion). For thin preparations like tissue cultures, oil objectives are best. Using an upright microscope, float a cover slip on the aqueous bath, and use immersion oil between the top of the cover slip and the objective.

2.4. If deconvolution software is used, the vendor might provide predetermined point spread functions (PSFs) for certain objectives. If no such information is provided, or if an unsupported objective is chosen, determine the PSF by imaging fluorescent microdots or similar diffraction-limited objects [7,8].

3. Establish the desired depth of field and number of images desired per time-lapse frame

3.1. Select the total depth of field so that moving or interacting structures of interest do not disappear or go out of focus as they move in the z-direction. The z-field should slightly exceed the vertical dimension of the cell or cell process being imaged. For vertebrate motor terminals, 15-20 µm is typical.

3.2. Calculate the z-axis step needed for each increment of focus. Resolution along the z-axis varies depending upon properties of the objective; it is about one-fourth of the x-y plane resolution. If either confocal imaging or deconvolution software is used, z-axis resolution is improved. Improve final resolution by deliberate oversampling in the z-direction [5], but see also 3.3 below. A typical step interval is in the range from 400 to 1500 nm for 40-100X objectives. Light should be shuttered off between each z-step image.

3.3. Select the number of images per z-stack, namely the desired depth of field divided by the step interval. Time to complete a typical z-stack is 1-10 sec. Fast-moving objects (100 nm/sec) can appear blurred in a 3D image stack because each image plane corresponds to a slightly different time point. Also, each additional image contributes to photobleaching and possible phototoxicity (see Discussion). If either situation pertains, choose a larger z-step to collect fewer images per stack. Compensate later using image interpolation software (6.3 below).

4. Select the time-lapse frame rate

Choose by experimentation a rate that is just adequate to smoothly resolve changes with time such as movement, interactions or fusion events [9]. Undersampling can produce motion artifacts (sampling errors), while oversampling unnecessarily increases exposure to light. Collect either a single long sequence or several repeated sequences from the same preparation, each with a relatively small total time interval (*e.g.,* 10 time points x 30 sec interval = 5 min). If possible, view the preparation with continuous epifluorescence illumination to roughly estimate movement velocities, including drift of the entire preparation if present.

5. Complete all live imaging for a particular preparation

Reptilian preparations typically remain robust for ~1-2 hr, longer if cooled.

6. Analyze data according to desired use

6.1. Retain all raw data files. While digital storage is usually not a problem, processing time is significant even with fast personal computers or workstations. For this reason, crop images into a region of interest [ROI]. Assure that the entire region of interest is in the cropped window during the entire time course.

6.2. Deconvolve image stacks using appropriate software and the correct point spread function. Confirm that resolution is improved and that no artifact has been generated by the deconvolution algorithm. Figure 3 shows example images.

6.3. Use an interpolation algorithm to expand z-axis apparent resolution. A 6X expansion from an actual 1.5 μm image plane separation to an apparent 0.25 μm separation is suggested. Alternatively, use some combination of oversampling (3.2) and interpolation.

6.4. Perform contrast, brightness, noise filtering, photobleaching and other typical image processing adjustments if desired. Image manipulation standards dictate that for most scientific work, it is appropriate that the same corrections be applied to all images, both within a stack and at different time points. The consequence of a particular adjustment should be assessed among all images [9].

6.5. Select a particular display mode for export of data. The most straightforward display is stereo video using either red-green or red-blue anaglyphs (examples in Fig. 3), stereo pairs, or alternating left/right eye frames with shutter glasses. Anaglyphs are limited to single-color. Enhance apparent image depth if desired to improve z-axis visual resolution.

6.6. Experiment with various filtering and image enhancement techniques. For example, Laplacian filtering is useful to increase contrast of small structures above background. The brightness of pixels at the center of a running window is enhanced while brightness of surrounding areas is reduced. Note that some filtering methods do not work well in combination.

6.7. Apply drift correction if there is substantial movement of the preparation over time. Such movement is common in living muscle, even with drugs added to suppress action potentials and synaptic potentials. A pixel registration algorithm (*e.g.*, IMARIS, Adobe AfterEffects,

TurboReg plugin for ImageJ) aligns time-lapse images and can reduce, but usually not completely eliminate, such motion.

Representative results

Data shown are from snake neuromuscular terminals (see low and high magnification views in Fig. 3; the endocytic dye (FM1-43) uptake creates a haze that fills each bouton) and, in particular, macroendosomes (MEs) and acidic endosomes (AEs) within these terminals [5]. MEs are created by bulk endocytosis during neural activity [10] and their number declines exponentially with time after activity has ceased [6]. Use of 4D live imaging was to determine if MEs move towards the plasma membrane and quickly disappear, suggesting that their number decreases because some of them exocytose. All such MEs were present at one time point (and those before) and completely gone at the next time point (and those after). Thus the time required for disappearance was less than our frame interval (as short as 30 sec) and consistent with exocytosis. An alternative theory, not supported by 4D data, is that MEs slowly dissipate via budding of vesicles until they vanish. Vesicle budding occurs [6], but at least some MEs are exocytosed either during or after budding [5]. Figure 1 and Movie S1 illustrate the disappearance of a ME between two time-lapse frames. MEs destined to vanish did not change shape or brightness over two or more frames (N = 16), which would have been consistent with slow dissipation during the time period of the movie (beginning several min after brief stimulation). ME disappearance was observed previously in conventional time-lapse, but it was possible that the structures had simply left the plane of focus. Moreover, viewing from a variety of perspectives (Movie S1) confirmed that the ME disappearance was sudden. With conventional live imaging (looking from a single perspective, e.g. the x-y image plane) noise artifacts limit the investigator's ability to confirm that a structure was unchanged. Such artifacts are often present in one view but not others.

Electron microscopy studies showed that endosomes in motor terminals are small in size, near the diffraction limit of light [10]. Hence fusion of these endosomes cannot be absolutely distinguished from close spatial association at light level. MEs were labeled with FM1-43 (green-fluorescing), and AEs with a lysosomal vital dye (red-fluorescing). Structures that fluoresced in both colors (appearing yellow) were occasionally noted in 4D image records. Using appropriate software, views of these double-labeled and therefore apparently fused endosomes from a variety of perspective were generated in order to examine the coincidence of the two labels in voxels near and within the apparent structure. Figure 2 shows a putative fused AE-ME as viewed from three orthogonal directions. One view is the x-y plane; the other views are orthogonal to that plane and to each other. Using this method it was found that voxels either overlapped, as in this example, or that they clearly didn't in one or more viewing planes. Movie S2 shows the same putative fused AE-ME presented as an interactive virtual reality display (fully rotatable in 3 dimensions).

Digital deconvolution is a useful tool to enhance resolution of 3D as well as 4D images, from both conventional and confocal microscopes [11]. Deconvolution is a numerical, iterative process that compensates, to some extent, for limited spatial resolution of the objective used. This resolution is characterized as the objective's point spread function—the 3D volume occupied by the image of an infinitesimal point. In theory, deconvolution restores the image that would result if the objective were perfect. Figure 3 is a 3D volume view of two different data sets, each

displayed as a pair of red-green anaglyphs. The right images show typical improvement in sharpness after digital deconvolution of the image stack.

Discussion

The most critical aspect of 4D imaging is management of the duration and intensity of light exposure. Photobleaching decreases image signal-to-noise ratio and can be problematic or not depending on various factors, including choice of fluorophores. Non-specific damage to living tissue (phototoxicity) is related to photobleaching, and can sometimes be identified using fluorescent probes designed for the purpose [2, 12] or by examination of morphology with suitable brightfield optics, such as differential interference contrast (DIC).

It is possible, however, to induce a physiological effect that occurs at light levels well below the magnitude of those associated with photobleaching and phototoxicity. For example, in early experiments using multiple preparations, each fixed at a different time after stimulation, the rate at which MEs disappeared with time after a brief stimulation was measured [6]. As is standard practice to minimize fading, preparations were kept in the dark until they were viewed. When similar experiments were performed using 4D live imaging, many more MEs remained throughout the experiment (~20-60 min) and did not disappear. Use of the same live imaging protocol, except not actually recording and thereby limiting light exposure to a single 3D frame at the beginning and a single frame at the end, restored the expected overall rate of endosome disappearance to that of fixed preparations.

Further troubleshooting indicated that the rate of disappearance was in part, inversely and monotonically related to total light exposure during an experiment. After unsuccessful attempts to reduce exposure via conventional and multiphoton confocal optics, the problem was finally resolved by purchase of a ~3X more sensitive camera (Table 1). It is recommended that similar comparisons be made if possible, depending on the user's application. If some process or transition is being documented over time, confirm that there is no modification attributable to intensity or total duration of illumination. Phototoxicity and photobleaching are dye-specific. For example, we saw no significant fading after repeated 4D imaging of one terminal (350 individual light exposures over 30 min) using FM1-43. The same imaging protocol using a similar dye, SGC5 (Table 1), however, resulted in some fading and, presumably, phototoxicity as well (data not shown).

With the exception of improvements provided by digital deconvolution (and confocal optics if used; not shown), the straightforward method described provides no "super" resolution. However, the method has the advantage of improving practical or intuitive resolution when viewing living structures, particularly moving ones, whose size is at or slightly larger than the diffraction limit. The ability to view objects from various perspectives, including presentation in each of three orthogonal planes, guides the investigator as to whether a structure actually exists above the noise level and whether it is labeled by one or more fluorophores. For example, in experiments designed to quantitate the number and size of MEs and AEs, it was possible to confirm that the structure was visible from various perspectives and that each dimension (and hence its three-dimensional volume) was within the range typical for that structure. The location of a structure is also better defined, for example, within an axon, nerve terminal etc. rather than above or below it.

In contrast, when the field of view was confined either to a single 2D image plane or to a single projection of 3D data, putative structures often proved to be "noise." Similarly, putative double-labeled structures often proved to be two structures that appeared coincident from one viewing perspective (or two) but not from all. Visualization from three orthogonal directions provides a standard and routine criterion for assessing the existence, size, and labeling characteristics of endosomes or similar structures for use in quantitative and statistical analyses.

The methodology described is practical in that a conventional video microscope of the type found in many laboratories can be used. The instrumentation is versatile rather than specialized, and relatively inexpensive. While software specific to a particular microscope was used here, open-source software for both image acquisition and data processing can produce similar results [13]. Techniques (including deconvolution) are applicable to single- and multi-photon confocal imaging with similar advantages, as long as light exposure is tolerated by the living preparation used. In the future, design improvements will further enhance the utility 4D live imaging. These include, primarily, hardware that enhances the speed of focusing and excitation/emission filter changing, and availability of even more sensitive video cameras.

Acknowledgements

This work was supported by the U.S. National Institutes of Health Grant NS-024572 (to R.S.W.).

Disclosures

The authors declare that they have no competing financial interests.

Table of specific reagents/equipment

See attached spreadsheet.

References

1. Frigault, M. M., Lacoste, J., Swift, J. L., & Brown, C. M. Live-cell microscopy–tips and tools. *J. Cell Sci.* **122(6)**: 753-767 (2009).
2. Tinevez, J.-Y., *et al.* A quantitative method for measuring phototoxicity of a live cell imaging microscope. *Meth. Enzymology* **506**: 291-309 (2012).
3. Dunn, K.W., Kamocka, M.M., & McDonald, J. H. A practical guide to evaluating colocalization in biological microscopy. *Am. J. Physiol. Cell Physiol.* **300**: C723–C742 (2011).
4. Snapp, E. L., & Hegde, R. S. Rational design and evaluation of FRET experiments to measure protein proximities in cells. *Current Protocols in Cell Biology* **17**: 17.9.1-17.9.20 (2006).
5. Stewart, R. S., Teng, H., & Wilkinson, R. S. "Late" macroendosomes and acidic endosomes in vertebrate motor nerve terminals. *J. Comp. Neurol.* **520**: 4275-4293 (2012).
6. Teng, H., Lin, M. Y., & Wilkinson, R. S. Macroendocytosis and endosome processing in snake motor boutons *J. Physiol.* **582.1**: 243–262 (2007).

7. McNally, J.G., Karpova, T., Cooper, J., & Conchello, J. A. Three-dimensional imaging by deconvolution microscopy. *METHODS* **19**: 373-385 (1999).
8. Swedlow, J. R., & Platani, M. Live cell imaging using wide-field microscopy and deconvolution. *Cell Structure and Function* **27**: 335-341 (2002).
9. Cromey, D. W. Digital images are data: and should be treated as such. In: Cell imaging techniques: methods and protocols (D. J. Taatjes and J. Roth, eds). *Methods in Molecular Biology* **931**: pp. 1-28 (2013).
10. Teng, H., & Wilkinson, R. S. Clathrin-mediated endocytosis near active zones in snake motor terminals. *J. Neurosci.* **20(21)**: 7986-7993 (2000).
11. Teng, H., Cole, J. C., Roberts, R. L., & Wilkinson, R. S. Endocytic active zones: hot spots for endocytosis in vertebrate nerve terminals. *J. Neurosci.* **19(12)**: 4855-4866 (1999).
12. Tan, T.T.T., Khaw, C., & Ng, M.M.L. Challenges and recent advances in live cell bioimaging. *Microscopy: Science, Technology, Applications and Education* (A. Mendez-Vilas & J. Diaz, eds.) pp. 1495-1505 (2010).
13. Verbrugghe, K. J. C., & Chan, R. C. Imaging *C. elegans* embryos using an epifluorescent microscope and open source software. *J. Vis. Exp*. **49**, e2625, doi:10.3791/2625 (2011).

Figure legends

Figure 1. A macroendosome (ME) disappears after apparent contact with plasma membrane.
A snake nerve-muscle preparation was electrically stimulated in the presence of FM1-43. Data were collected using 4D imaging as described in Methods and are displayed as "3D Volume views" at six time points (60 sec interval) to illustrate the Z-depth of a single bouton. An ME can be observed moving away from the Y axis (red dashed line) over several frames before disappearing between time points 5 and 6. Just before disappearance, the ME appears to be located at the bouton's plasma membrane (delineated by FM1-43 vesicle staining or "haze;" see Fig. 3 legend). The ME did not re-appear in later time-points (data not shown; see Movie S1). Scale bar, 2 μm.

MOVIE S1: 4D time-lapse views of ME disappearance.
The same raw data set displayed in Fig. 1 is shown as a "4D volume view" at a lower magnification. The original time-lapse sequence comprises 8 time-points, each separated by 60 sec; playback is at 4 frames/sec. The sequence is briefly paused at the beginning of each of 24 3D rotation steps about the y-axis (15 degrees/step) to draw attention to the soon-to-be disappearing ME (red arrow). Note that the ME is visible, approaches the edge of the bouton (plasma membrane), disappears, and doesn't return, in all viewing perspectives. Because of lower z-axis resolution compared to x-y resolution, the shape of the ME appears to lengthen and shorten depending on viewing perspective. Images were exported as a QuickTime movie, and annotated using QuickTime Pro. Scale bar, 2 μm.

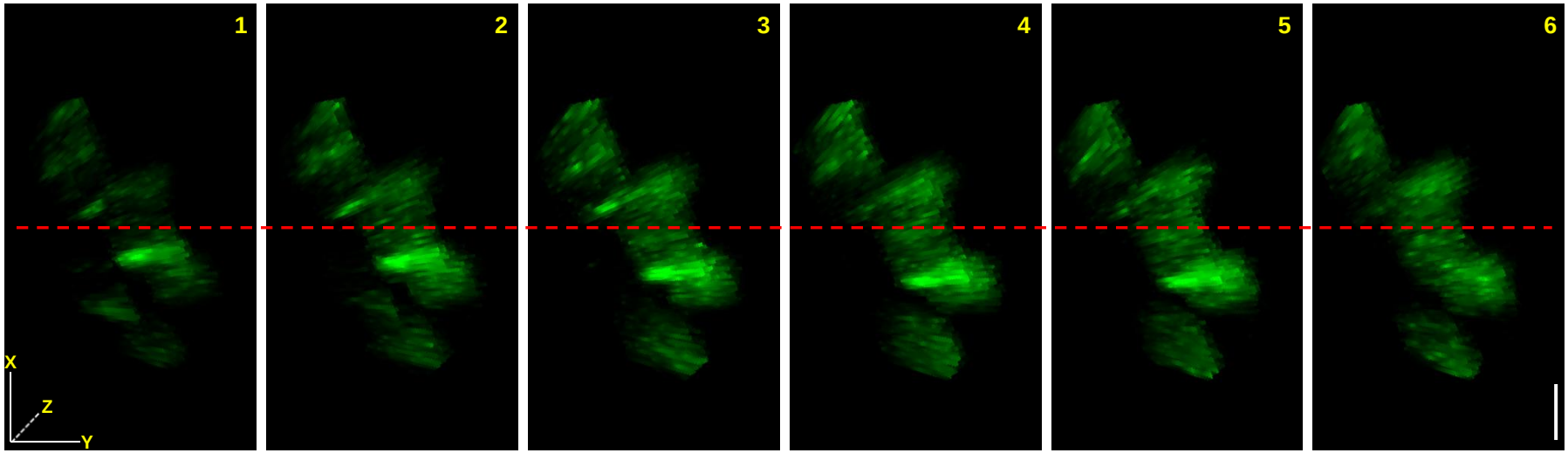
Figure 2. Orthogonal views of a putative fused endosome.

A nerve muscle preparation was sequentially stained with a lysosomal vital dye (red) to label AEs and FM1-43 (green) to label MEs using 0.5 M sucrose stimulation as described in Methods. Data from one time point of a 4D movie are displayed as 3D volume views in three orientations. At top (panels 1-3) is the conventional view from above (x-y plane). At middle (panels 4-6) is a view perpendicular to the x-y plane and in the (arbitrary) direction of the large red arrow. At bottom (panels 7-9) is a view mutually perpendicular to both views above, in the direction of the large blue arrow. An ME (green) is marked by a green arrow; two AEs (red) are marked by red arrows. An endosome containing both dyes (yellow) is marked by a yellow arrow in panels 3, 6, and 9. Note that overlap of red and green is equally complete in all three orthogonal projections. Scale bar, 2 μm.

MOVIE S2: Interactive rotation of image containing MEs and acidic endosomes AEs.
The data set of Fig. 2 is shown configured as a fully rotatable 3D image (QuickTime Virtual Reality format; use the mouse to rotate the image for viewing from any direction). The putative fused ME/AE remains yellow from all perspectives.

Figure 3. Deconvolution enhances 3D spatial resolution.
Two typical snake nerve terminals, shown at low (top) and high (bottom) magnification respectively and stained during stimulation with FM1-43. FM1-43 background "haze," due to labeling of individual 50 nm vesicles, shows the shape of terminal boutons within which MEs are confined. Data are displayed as Red-Green anaglyphs of 3D volume views (depth can be visualized with red-green or red-blue glasses; red on left eye; note axon at upper left descends into the plane of the terminal from above). Left panels: raw data; Right panels: data after deconvolution. Note the significant improvement in resolution of MEs. Top panels: Electrical stimulation; entire terminal shown (~30 x 50 μm) (same raw data as in Fig. 1 and Movie S1; scale bar, 10 μm). Bottom panels: KCl stimulation; magnified to show four individual boutons (~3 x 5 μm; scale bar, 2 μm).

*Figure
Click here to download Figure: Fig1-092713.pptx



*Figure

Click here to download Figure: Fig2-092713.pptx

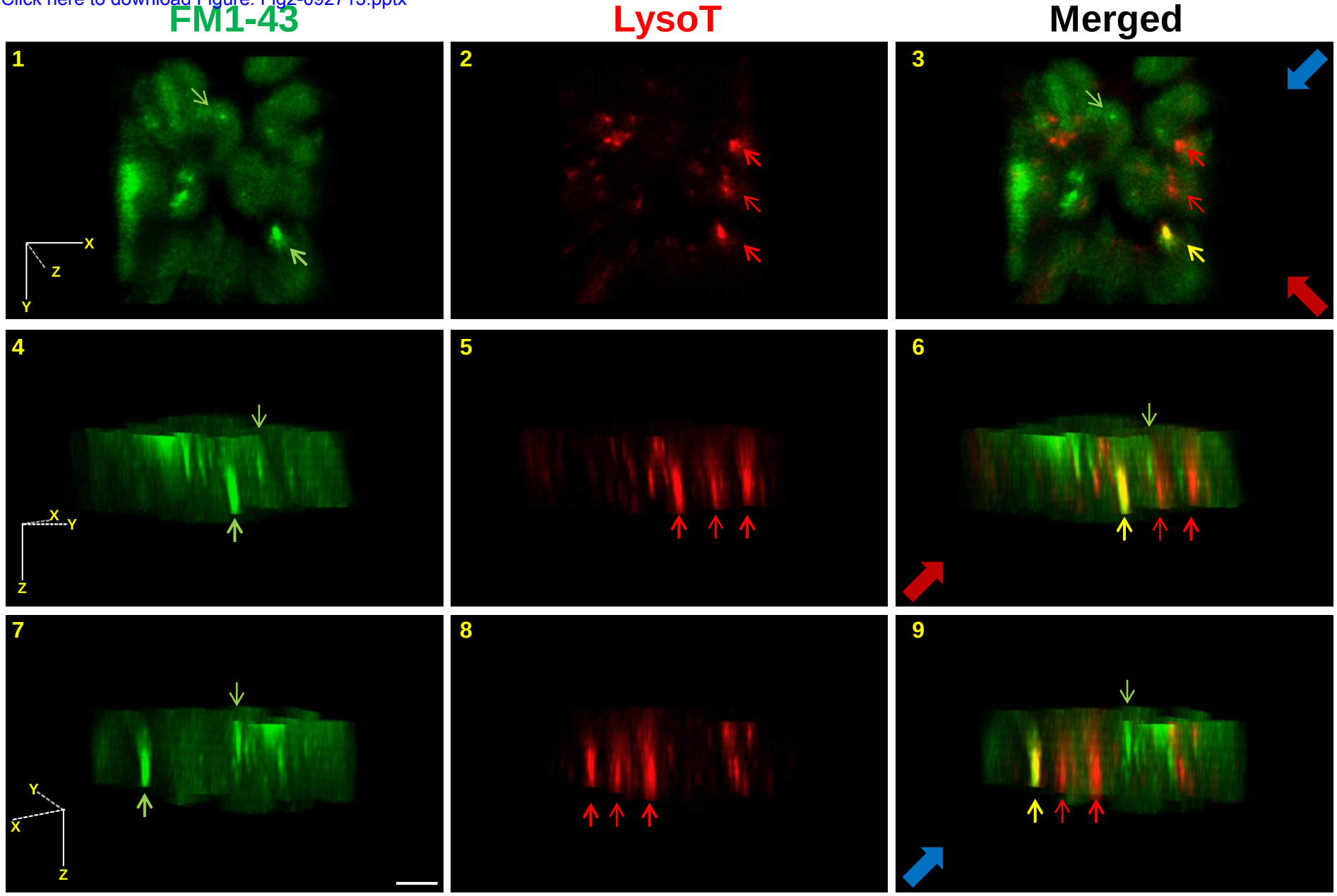
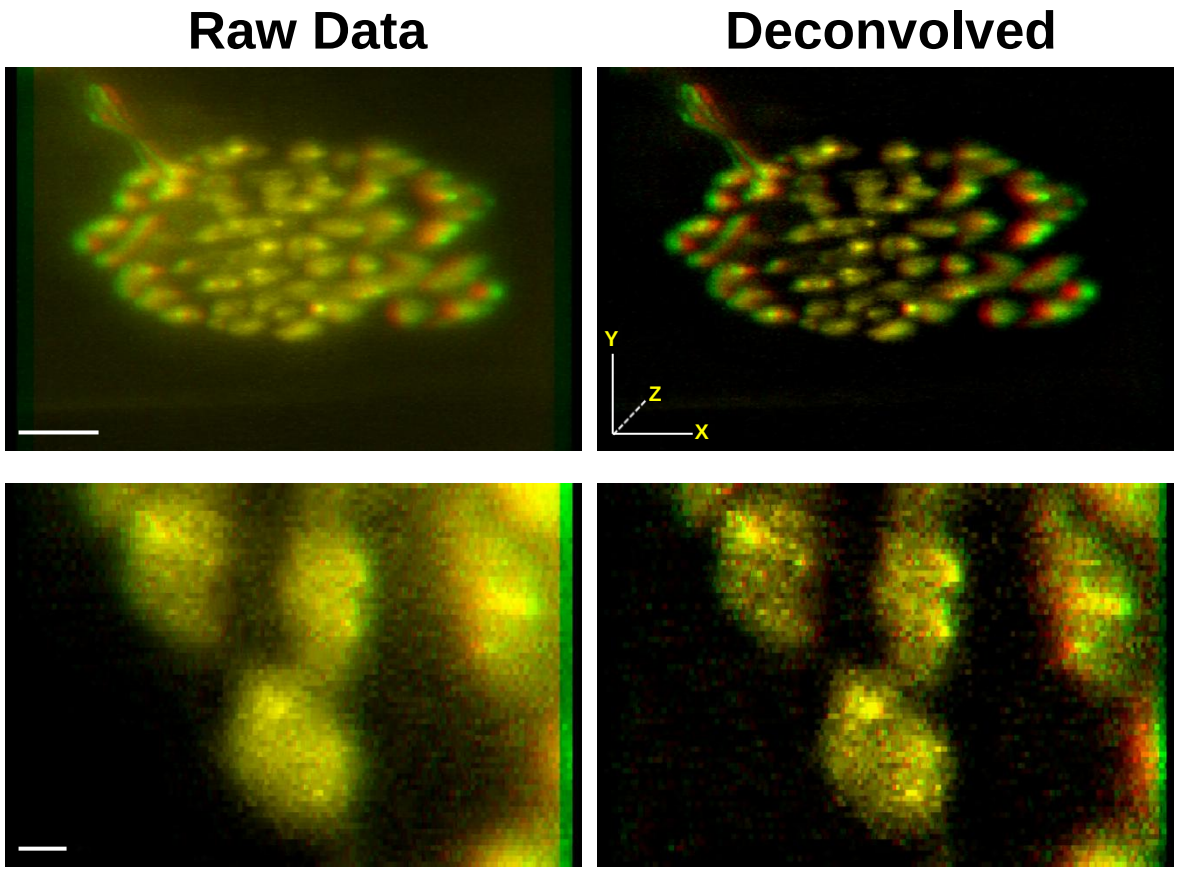


*Figure
Click here to download Figure: Fig3-092713.pptx



*Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Table-spreadsheet-v7-092413.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments
Reagents:			
SGC5	Biotium [Hayward, CA]	70057	Final conc:10 μM
FM1-43FX	Invitrogen [Carlsbad, CA]	F35335	Final conc:7 μM
LysoTracker Red	Invitrogen [Carlsbad, CA]	L7528	Final conc:0.2 μM
Solutions:			
Reptilian Ringers pH 7.2			
NaCl	145 mM		
KCl	2.5 mM		
$CaCl_2$	3.6 mM		
$MgSO_4$	1.8 mM		
KH_2PO_4 (Dibasic)	1.0 mM		
HEPES	5.0 mM		
High KCL Reptilian Ringers pH 7.2			
NaCl	86 mM		
KCl	60 mM		
$CaCl_2$	3.6 mM		
$MgSO_4$	1.8 mM		
KH_2PO_4 (Dibasic)	1.0 mM		
HEPES	5.0 mM		
High Sucrose Ringers pH 7.2			
NaCl	145 mM		
KCl	2. 5 mM		
$CaCl_2$	3.6 mM		
$MgSO_4$	1.8 mM		
KH_2PO_4 (Dibasic)	1.0 mM		
HEPES	5.0 mM		

Sucrose	0.5 M (17.1 gm/50 mL)

Equipment:

Name	Company	Comments	Comments(website)
Axioplan 200 inverted mic	Carl Zeiss [Thornwood, NY]		www.zeiss.com
N-Achroplan 63X water ob	Carl Zeiss [Thornwood, NY]		www.zeiss.com
DG4 combination light sou	Sutter instruments [Novato	175W Xenon arc lamp	www.sutter.com
Lambda 10-2 emission filt	Sutter instruments [Novato, CA]		www.sutter.com
Sensicam CCD camera	Cooke Instruments [Tonawanda, NY]		www.cookecorp.com
Cascade 512 CCD camera	Photometrics [Tucson, AZ]		www.photometrics.com
Imaging dishes- made in-house-11cm dia.; 25 mm dia. #1 coverslip embedded; magnetic pins			

Software:

Name	Company	Comments	Comments(website)
Slidebook 5.0	Intelligent Imaging Innovat	Deconvolution; Drift correc	www.intelligent-imaging.com
IMARIS 7.5.2	Bitplane [South Windsor, C	Drift correction; 3D and 4D	www.bitplane.com
AfterEffects CS6	Adobe [San Jose, CA]	Drift correction	www.adobe.com
ImageJ 1.46	National Institutes of Healt	Multiple plugins available;	http://rsbweb.nih.gov/ij
Zeiss LSM	Carl Zeiss [Thornwood, NY	Stereo pair construction	www.zeiss.com

Animated Figure (video and/or .ai figure files)

Click here to download Animated Figure (video and/or .ai figure files): MOVIE-S1-073013.mov

Animated Figure (video and/or .ai figure files)

Click here to download Animated Figure (video and/or .ai figure files): MOVIE-S2-073013.mov

jove JOURNAL OF VISUALIZED EXPERIMENTS
17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Visualization of Endosome Dynamics in Living Nerve Terminals with Four-Dimensional Fluorescence Imaging

Author(s): Richard S. Stewart, Ilona M. Kiss, and Robert S. Wilkinson

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

jove JOURNAL OF VISUALIZED EXPERIMENTS
17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name: Robert S Wilkinson

Department: Dept. of Cell Biology and Physiology

Institution: Washington University School of Medicine

Article Title: Visualization of Endosome Dynamics in Living Mice Tumors with Four-Dimensional Fluorescence Imaging

Signature: Robert S Wilkinson Date: 30 July 2013

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy as a PDF to the *JoVE* submission site upon manuscript submission (preferred);
2) Fax the document to +1.866.381.2236; or
3) Mail the document to *JoVE* / Attn: *JoVE* Editorial / 17 Sellers St / Cambridge, MA 02139

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use):

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.

Screenshots-powerPoint-part3

Click here to download Supplemental File (as requested by JoVE): Protocol-part3-screenshots.pptx

Screenshots-powerPoint-part6

Click here to download Supplemental File (as requested by JoVE): Protocol-part6-screenshots.pptx

Screenshots-WordFile-part3

Click here to download Supplemental File (as requested by JoVE): JOVE-imaging-protocol-part3-resaved-081213.doc

Screenshots-WordFile-part6

Click here to download Supplemental File (as requested by JoVE): JOVE-SB-editing-part6-resaved-081213.doc