TITLE: Live imaging of dense-core vesicles in primary cultured hippocampal neurons

Authors:

David M. Kwinter and Michael A. Silverman

Authors: institution(s)/affiliation(s) for each author:

David Kwinter

Department of Biological Sciences

Simon Fraser University, Burnaby, BC, Canada

david@kwinter.ca

Michael Silverman

Department of Biological Sciences

Simon Fraser University, Burnaby, BC, Canada

masilver@sfu.ca

Corresponding author: Michael Silverman

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Short Abstract:

Live cell imaging is of particular utility when studying the dynamics of organelle trafficking. Here we describe a protocol for live imaging of dense-core vesicles in cultured neurons using wide-field fluorescence microscopy. This protocol is flexible and can be adapted to image other organelles such as mitochondria, endosomes, and peroxisomes.

Long Abstract:

Observing and characterizing dynamic cellular processes can yield important information about cellular activity that cannot be gained from static images. Vital fluorescent probes, particularly green fluorescent protein (GFP) have revolutionized cell biology stemming from the ability to label specific intracellular compartments and cellular structures. For example, the live imaging of GFP (and its spectral variants) chimeras have allowed for a dynamic analysis of the cytoskeleton, organelle transport, and membrane dynamics in a multitude of organisms and cell types [1-3]. Although live imaging has become prevalent, this approach still poses many technical challenges, particularly in primary cultured neurons. One challenge is the expression of GFP-tagged proteins in post-mitotic neurons; the other is the ability to capture fluorescent images while minimizing phototoxicity, photobleaching, and maintaining general cell health. Here we provide a protocol that describes a lipid-based transfection method that yields a relatively low transfection rate (~0.5%), however is ideal for the imaging of fully polarized neurons. A low transfection rate is essential so that single axons and dendrites can be characterized as to their orientation to the cell body to confirm directionality of transport, i.e., anterograde v. retrograde. Our approach to imaging GFP expressing neurons relies on a standard wide-field fluorescent microscope outfitted with a CCD camera, image capture software, and a heated imaging chamber. We have imaged a wide variety of organelles or structures, for example, dense-core vesicles, mitochondria, growth cones, and actin without any special optics or excitation requirements other than a fluorescent light source. Additionally, spectrally-distinct, fluorescently labeled proteins, e.g., GFP and dsRed-tagged proteins, can be visualized near simultaneously to characterize co-transport or other coordinated cellular events. The imaging approach described here is flexible for a variety

of imaging applications and can be adopted by a laboratory for relatively little cost provided a microscope is available.

Part 1: Transfection of neurons using Lipofectamine 2000 (Invitrogen)

Equipment set-up:

Rat or mouse hippocampal neurons are cultured according to Kaech and Banker, 2006 [4]. The typical cell density used for transfections is 250,000 cells/6-cm dish. Required reagents and equipment include 50 mM kynurenic acid, regular benchtop tube holder, a benchtop cooler (best to keep Lipofectamine reagent cold), Lipofectamine reagent, MEM, microfuge tubes, micropipetters, and sterile forceps.

Procedure:

1. For each transfection label two 1.5 mL tubes; one to contain the plasmid DNA, one to contain the transfection reagent. The following incubations can proceed at room temperature.
    1. Combine 1.0 µg of plasmid DNA with 100 µL MEM in one tube (without supplements of any type; MEM does not need to be temperature of pH equilibrated).
    2. Combine 6.0 µL Lipofectamine with 100 µL MEM in the other 1.5 mL tube.
        1. No adjustments for double transfections are required even though the Lipofectamine:DNA ratio will be halved in such cases. The ratio of Lipofectamine:DNA is still within the manufacturer's suggestion. It is best to test 0.5-1.0 µg for each plasmid for optimal expression.
        2. To preserve the shelf life of the Lipofectamine reagent, it should be removed from the refrigerator for as short a time as possible and placed in a benchtop cooler when not in use. Total time out of the refrigerator should be kept to a minimum.

2. Incubate the tubes for 5 minutes at room temperature.
3. Transfer the DNA-in-MEM solution into the lipid tube, and gently mix with pipette, then incubate for 30 minutes at room temperature.
4. After 25 minutes, before flipping coverslips, add 60 µL of 50 mM kynurenic acid to minimize excitoxic damage to the dish of cultured neurons.
    1. Carefully flip coverslips feet-side-up using sterile forceps, and arrange so they do not overlap. Be careful not to scrape the neuron-coated surface of the coverslip or the glia-coated surface of the dish.

5. Take approximately 0.5 mL of medium from the 6-cm dish and gently mix with DNA in tube, transfer the DNA solution back to the dish dripping evenly onto surface of the medium.
6. Don't swirl, but gently slide dish back and forth in one direction, then stop and repeat in perpendicular direction to evenly distribute the DNA-Lipofectamine complexes. Incubate for 90 minutes in the tissue culture incubator (37˚C, 5% CO_2).
7. Flip coverslips feet-side-down, and arrange so they do not overlap. Allow expression to proceed for the desired time, typically overnight to 48 hours.

Part 2: Live imaging of GFP-tagged dense-core vesicles in cultured hippocampal neurons

Equipment set-up:

1. Imaging of live cells requires a fluorescence microscope equipped with a CCD camera; we use a Leica DMI 6000B inverted microscope equipped with the Leica variable, fluorescent lamp. Also required is an imaging chamber with temperature controller and an objective heater. We use the Warner Instruments RC-21BR modified for an 18-mm coverslip in addition to a platform heater (cat. #PH2) and temperature controller (cat. #TC324B). The chamber contains no open ports, a modification that can be included when ordering the chamber. An objective heater is highly recommended to help maintain the temperature of the coverslip and minimize changes in focus due to temperature fluctuations. Images are acquired with a Hamamatsu Orca-ER using Metamorph (Molecular Devices) software including the 'streaming' mode of acquisition drop-in. Note- We do not use a climate chamber that encloses the entire microscope. This addition is expensive and not necessary for the short-term imaging described here.
2. Other equipment and reagents include freshly prepared live imaging medium (1X Hanks with Ca^{2+} and Mg^{2+}, 0.6% Glucose, and 10mM HEPES), additional 18-mm glass coverslips, sterile forceps, non-sterile

forceps, Kimwipes, vacuum grease, and a syringe (without needle, or with modified wide-bore needle) with which to apply grease.

Procedure:

1. Prepare fresh live imaging medium. It is best to prepare enough for no more than one week at a time. Approximately 5-10 mL are used per dish of neurons.
2. Start microscope, camera, fluorescent lamp, and image acquisition software. Also turn on power to the chamber platform and objective heaters.
3. Prepare the imaging chamber.
    1. The Warner chamber includes a Teflon insert for the mounting of coverslips. For ease of manipulation, label one side of the insert "top" with a permanent marker.
    2. Apply a ring of grease to the groove surrounding the hole in the side of the chamber labeled "top". Avoid using excess as it will enter the chamber and reduce the observable area.
    3. Using forceps, attach a clean, unused 18-cm coverslip to the "top" side of the chamber, apply gentle pressure to the coverslip at its edges to create a tight seal within the recessed groove of the chamber.
    4. Turn the chamber over and apply a similar ring of grease to the groove on the opposite (bottom) side of the chamber.
    5. Place the prepared chamber—bottom side up—on the lid of a 50-mL tube, which is an ideal chamber holder.
    6. Add 750 µL of imaging medium to the chamber. This is an excessive amount, but the grease should prevent the medium from spilling over and the excess will help prevent bubbles from being trapped when the cell-covered coverslip is applied.
    7. Maintain the prepared chamber in the tissue culture incubator until needed. Prolonged incubations, ~ 1 hour should be avoided as the imaging medium will evaporate and change in composition.

4. Perform the final chamber assembly and live cell imaging.

    1. Move the prepared chamber and a dish of transfected coverslips from the incubator to the tissue culture hood.
    2. The final chamber assembly should be performed quickly to ensure the cells remain immersed in medium and at 37˚C continuously.
    3. Using sterile forceps, carefully remove one coverslip from the transfected dish, touch the edge of the coverslip to a Kimwipe to draw away growth medium.
    4. Place the coverslip neuron-side-down onto the prepared chamber. Touch one side of the coverslip to the grease first and apply pressure around the edges to bring the coverslip flat without trapping bubbles. Excess imaging medium will spill out as expected.
    5. Wipe off excess imaging medium, but be careful not to slide the coverslips out of position.
    6. Move the chamber to the pre-heated platform heater and fasten the chamber in place.
    7. Transfer the chamber to the stage.
    8. To reduce photobleaching and photoxicity, adjust the lamp to the minimum amount of intensity necessary to find transfected cells.
    9. Find a cell of interest with a low magnification oil objective (40X). It is beneficial to stick to a planned search pattern to ensure maximal coverslip coverage and to avoid redundant image acquisitions, e.g., top left of coverslip, scanning up and down working to the right.
    10. Switch to a high magnification oil objective (60-100x) once a desired field has been located.
    11. Use "stream acquisition" or comparable function of your imaging software to record a video of fluorescently-labeled protein dynamics.
    12. Take a phase image and any other accompanying images (e.g. Soluble GFP) for later analysis and presentation.
    13. Save all images before exploring the coverslip further and recording the next video.
    14. Typically 3-5 videos from one coverslip is considered successful. Phototoxicity and general cell health should be kept in mind as transport is reduced in damaged cells. Cell health can be monitored by observation using phase microscopy. Typically recordings are performed for approximately 30 minutes per coverslip.

Part 7: Representative Results:

Live cell imaging observations typically consist of videos (also known as "stacks" of images) that show movement of fluorescently-tagged organelles within the field of view (Figure 1A). Accompanying each video are often supporting images that illustrate orientation of the field of view with respect to the cell body, level of protein expression and/or the health of the cell. Videos of transport can be subjected to quantitative analysis by transformation to kymographs (Figure 1B). Kymographs are images that illustrate particle movement by juxtaposing line scans where each time point is represented by a column in the kymograph. Figure 2 demonstrates how two particles moving in opposite direction are represented in a kymograph. Further analysis of kymographs can provide information about particle flux, velocity, run length, reversals, and stationary particles.

Discussion:

Live cell imaging is a challenging, but powerful technique for the direct observation of organelle transport in cultured neurons. Difficulties can arise upstream of the procedure with poor neuron health due to culture complications. Therefore, cell health (for examples see ref. 4) should be assessed before and after transfection by observing the coverslips while in growth medium using a tissue culture light microscope. The process of transferring neuron-containing coverslips to the imaging chamber can be stressful to the cells, thus it is important to exercise care when manipulating the coverslips. It can be common for healthy-looking cells to show no transport due to delays in the procedure, or incomplete pre-heating of equipment or equilibration of the imaging media. For transport analysis the axonal and dendritic orientation must be known, i.e. distinguishing anterograde and retrograde transport. It is therefore critical to be sure that the location of a cell body is determined and that a continuous neuronal segment can be seen connecting the region of interest to the cell body. Often saving overlapping images of soluble GFP, or informatively naming the saved video file, is sufficient to ascertain the processes' orientation for analyses, e.g., "Cell1CellBodyUpperLeft".

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Table of specific reagents and equipment:

Name of the reagent	Company	Catalogue number	Comments (optional)
MEM- Eagle with Earle salts and L-glutamine	Mediatech	10-010-CV	
Lipofectamine 2000	Invitrogen	11668-027	Transfection reagent
10X Hanks with Ca^{2+} and Mg^{2+}	Gibcoo/Invitrogen	14185-052	Live-imaging medium
1M HEPES	Gibcoo/Invitrogen	15630-130	Live-imaging medium

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4. Kaech, S. and G. Banker, *Culturing hippocampal neurons.* Nat Protoc, 2006. **1**(5): p. 2406-15.

Figures and Figure Legends

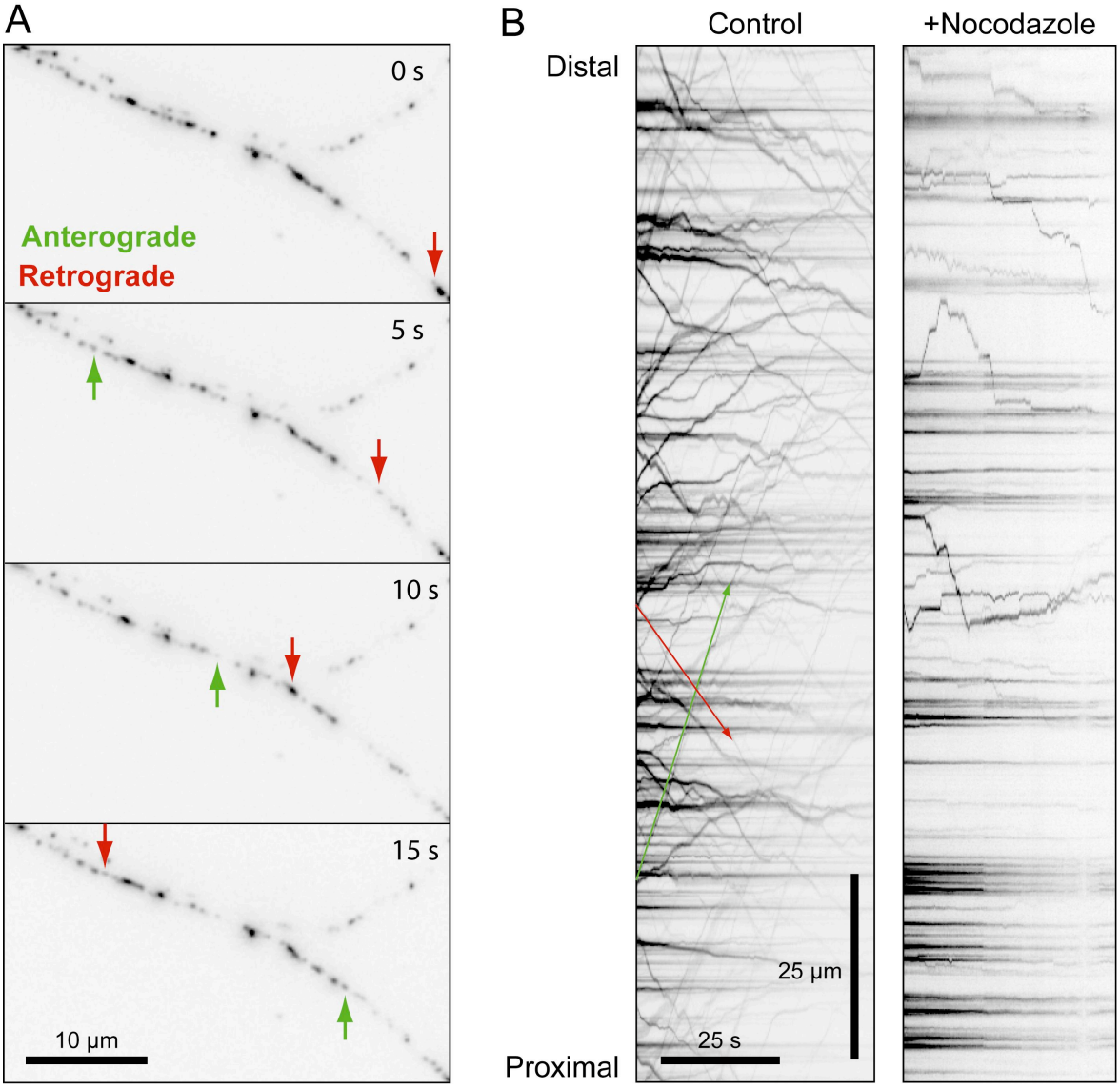

Figure 1. Representative Live Cell Imaging Observations. (A) Selected frames from a video of mCherry-tagged Tissue Plasminogen Activator (TPA) transport. Individual particles moving in the anterograde (green arrows) and retrograde (red arrows) directions are indicated. **(B)** Kymographs illustrating TPA-mCherry movement in the axon. Horizontal lines in kymographs represent stationary objects, while diagonal lines represent organelles moving away from (positive slope) or toward (negative slope) the cell body. The long green and red arrows show the runs corresponding to the particles in (A). Changes in transport due to the microtubule depolymerizing reagent, nocodazole, is also illustrated with a kymograph. Note the reduction in moving organelles by the absence of diagonal lines.