Video Article

Flash-and-Freeze: A Novel Technique to Capture Membrane Dynamics with Electron Microscopy

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Abstract

Cells constantly change their membrane architecture and protein distribution, but it is extremely difficult to visualize these events at a temporal and spatial resolution on the order of ms and nm, respectively. We have developed a time-resolved electron microscopy technique, "flash-and-freeze," that induces cellular events with optogenetics and visualizes the resulting membrane dynamics by freezing cells at defined time points after stimulation. To demonstrate this technique, we expressed channelrhodopsin, a light-sensitive cation channel, in mouse hippocampal neurons. A flash of light stimulates neuronal activity and induces neurotransmitter release from synaptic terminals through the fusion of synaptic vesicles. The optogenetic stimulation of neurons is coupled with high-pressure freezing to follow morphological changes during synaptic transmission. Using a commercial instrument, we captured the fusion of synaptic vesicles and the recovery of the synaptic vesicle membrane. To visualize the sequence of events, large datasets were generated and analyzed blindly, since morphological changes were followed in different cells over time. Nevertheless, flash-and-freeze allows the visualization of membrane dynamics in electron micrographs with ms temporal resolution.

Video Link

The video component of this article can be found at https://www.jove.com/video/55664/

Introduction

Visualizing membrane and protein dynamics within a cell is a key step towards understanding the cell biology of particular processes. Dynamic trafficking events can be captured using light or fluorescence microscopy. However, the subcellular context is largely missing in such images because subcellular structures cannot be completely "painted" by dyes or fluorescent probes and resolved spatially and spectrally[1,2]. On the other hand, while electron microscopy can delineate subcellular architecture in exquisite detail, it cannot capture cellular dynamics, because specimens must be fixed prior to imaging. Thus, it is typically not sufficient to completely understand cellular dynamics using only one imaging modality.

To overcome the limitations of light and electron microscopy, correlative microscopy techniques have been developed. Correlative Light and Electron Microscopy (CLEM) visualizes intracellular dynamics using light microscopy and underlying subcellular structures with electron microscopy. In CLEM, cells engaged in various processes, such as cytokinesis and endocytosis[3,4,5,6], are live-imaged and then processed for electron microscopy. Although CLEM captures certain aspects of intracellular dynamics, there are four factors that limit the utility of this approach. First, the temporal resolution is limited by how quickly the cells can be immobilized, which typically takes s - min due to the slow diffusion and reaction of fixatives[7]. Second, the subcellular architecture is observed *post facto*[8]; thus, the dynamic morphological changes cannot be captured using this approach. Third, the fluorescence and electron micrographs cannot be precisely aligned due to tissue shrinkage caused by dehydration during the sample preparation for electron microscopy[9,10]. Fourth, events like cytokinesis and endocytosis do not take place at the same time in every cell[5,11], and thus, a particular cell that is engaged in the event must be identified from a large population of cells. This process is often laborious. Thus, a new method is necessary to induce particular events in every cell and to capture the resulting cellular dynamics by the rapid immobilization of cells at defined time points.

Recently, several tools have been developed to induce particular cellular dynamics using light (optogenetics). Channelrhodopsin is a light-sensitive, non-selective cation channel isolated from *Chlamydomonas reinhardtii*[12,13]. When channelrhodopsin is expressed in neuronal membranes, a brief flash of light induces an influx of sodium ions into neurons and triggers an action potential[14,15]. The action potential then propagates into the synaptic terminals, where synaptic vesicles fuse within milliseconds[16,17,18] Therefore, channelrhodopsin induces neuronal

activity. To follow membrane dynamics at synaptic terminals, neurons must be immobilized at defined time points after stimulation with ms precision.

To capture membrane dynamics after inducing neuronal activity, we coupled light stimulation with high-pressure freezing[17,18,19]. High-pressure freezing allows for the near-instantaneous immobilization of cells with reduced ice crystal formation[20]. Ice crystals can rupture membranes and disrupt the subcellular architecture[21]. By varying the time intervals between stimulation and freezing, membrane trafficking within synaptic terminals was captured following the induction of an action potential.

Here, we demonstrate experimental procedures using a commercialized high-pressure freezer that couples a ms temporal control of light stimulation with high-pressure freezing. Unlike other instruments that require an external device to control light stimulation and freezing, light stimulation is fully integrated in this system and can be applied with ms precision[19]. This process involves multiple steps. 1) Mouse hippocampal neurons are cultured on sapphire disks and infected with lentivirus carrying an expression vector for channelrhodopsin[18]. 2) Neurons are stimulated and frozen at defined time points after stimulation. 3) The vitrified water is substituted with an organic solvent, while lipids and proteins are cross-linked by fixatives to preserve the intracellular architecture. 4) The samples are infiltrated and embedded in epoxy resin. 5) Ultrathin sections are collected using an ultramicrotome. 6) Thin sections are imaged on a transmission electron microscope. 7) Image acquisition and analysis are performed blindly with respect to time points or genotypes. Cellular dynamics can be determined through the reconstruction of time-resolved images[17,18]. Sample preparation (steps 2 - 5 above) requires a week, but the subsequent image analysis requires months to a year.

Protocol

All of the experiments were performed according to the rules and regulations of animal use by the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee (IACUC) at Johns Hopkins School of Medicine.

1. Isolation and Culture of Mouse Hippocampal Neurons

1. Dissect cortices from a postnatal day 0 - day 2 (P0 - 2) mouse brain[18]. Isolate the astrocytes from the cortices.
   NOTE: The mouse brain was isolated after decapitation. Astrocytes serve as a feeder layer for hippocampal neurons.
2. Treat the cortices with 800 µL of 0.05% Trypsin-EDTA for 15 min at 37 °C to dissociate the astrocytes.
3. Culture the astrocytes in a T-75 flask with 13 mL of DMEM containing 10% fetal bovine serum (FBS) and 0.2% penicillin-streptomycin for one week at 37 °C and 5% CO_2.
4. Place one acid-washed and sterilized 18 mm glass coverslip per well of a 12-well plate.
5. Briefly wash two 6 mm carbon-coated sapphire disks in 70% ethanol and place them on top of each glass coverslip.
6. Prepare Poly-D-Lysine (PDL) solution by mixing 3 mL of 17 nM acetic acid with 1 mL of rat tail collagen and 1 mL of PDL (1 mg/mL). Apply 200 µL of PDL solution to the sapphire disks and glass coverslips for 5 min at RT.
7. Remove the PDL solution and air dry. Prior to use, sterilize the plate as prepared in steps 1.4 - 1.6 for 30 min under ultraviolet light.
8. Seed the astrocytes from step 1.3 in 2 mL of DMEM at a density of $5x10^4$ cells/well in the plate as prepared in steps 1.4 - 1.6. Grow them at 37 °C and 5% CO_2 for one week.
9. Add 20 µL of fluoro-deoxyuridine (final concentration: 80 µM) to each well for at least a few h before culturing the neurons.
   NOTE: Fluoro-deoxyuridine stops astrocyte division.
10. Change the medium to 1.5 mL of neuronal basal medium (see the **Table of Materials**) containing 1% L-alanyl-L-glutamine (see the **Table of Materials**), 2% serum-free supplement for neuronal cells (see the **Table of Materials**), and 0.2% penicillin-streptomycin.
11. Prepare papain solution by adding 20 units of papain to 5 mL of enzyme solution (1.65 mM Cysteine, 1 mM $CaCl_2$, and 0.5 mM EDTA in DMEM). Acidify the solution by passing CO_2 gas for 20 min. Filter-sterilize with a 0.22 µm filter.
12. Harvest the brain from a P0 - 2 mouse[18]. Immediately transfer the brain to ice-cold Hanks'-balanced Salt Solution (HBSS). Dissect the hippocampi under a stereomicroscope, keeping the tissue submerged in HBSS.
13. Place the two hippocampi in 1 mL of the papain solution prepared in step 1.11. Incubate for 1 h on a thermomixer at 37 °C and 750 rpm.
14. Replace the papain with 1 mL of inactivating solution containing 2.5 mg of trypsin inhibitor and 0.5 mg of albumin per mL of DMEM. Incubate for 5 min at 37 °C. Aspirate off the inactivating solution.
15. Add 200 µL of neuronal basal medium (see the **Table of Materials**) to the isolated hippocampus. Triturate using a 200 µL pipette tip to dissociate the cells. Wait until the undissociated cells settle at the bottom. Carefully remove the medium with cells from the top.
16. Repeat step 1.15 3x. Pool all the dissociated cells in a new 1.5 mL centrifuge tube.
17. Count the number of cells using a hemocytometer. Plate neurons at a density of 6.5 x 10^4 cells/well on top of the astrocyte layer prepared in steps 1.1 - 1.10.
18. Infect the neurons with lentivirus expressing channelrhodopsin at DIV 3 (3 d *in vitro*)[18].
19. Perform flash-and-freeze on DIV 14, as described in steps 2.1 - 2.5, below.

2. Flash-and-Freeze

1. **Preparation of Fixative**
   1. Under a chemical hood, add the following substances to a conical tube to prepare the fixative: 2.5 mL of glutaraldehyde (10% stock in acetone), 0.25 g of osmium tetroxide, 0.25 mL of water, and 22.25 mL of anhydrous acetone.
      NOTE: The final concentration of each component should be 1% in acetone. Glutaraldehyde is added to preserve the protein structures during freeze-substitution. CAUTION: The acute toxicity of osmium tetroxide is high. Exposure to vapors could damage the cornea of the eye. It should be handled only in a certified chemical hood.
   2. Aliquot 1 mL of fixative into numbered cryogenic vials (2 mL). Keep the fixative frozen in liquid nitrogen until use.

NOTE: Osmium tetroxide and glutaraldehyde cross-react and precipitate; thus, once mixed, aliquot immediately, cap the tubes, and submerge the cryotubes in liquid nitrogen to freeze the fixative. Use a pencil to number the cryogenic vials, since acetone can wash off markers.

2. **Preparation of Physiological Saline**
    1. Make physiological saline solution by mixing Hepes (10 mM, pH 7.5), NaCl (140 mM), KCl (2.4 mM), and glucose (10 mM).
    NOTE: These values are final concentrations. Cryo-protectants are not used for monolayer cultures. However, a proper cryo-protectant is required for specimens thicker than 5 µm. The use of 20% BSA is typically recommended. Yeast paste and *E. coli OP50* can also be used as cryo-protectants for fly larvae or *C. elegans*.
    2. Add $CaCl_2$ at 4 mM and $MgCl_2$ at 1 mM final concentrations.
    NOTE: The concentrations of $CaCl_2$ and $MgCl_2$ vary depending on experiments. To ensure the capture of exocytic intermediates, 4 mM calcium is used for these particular experiments to increase the release probability of vesicles[18].
    3. Check the osmolarity using an osmometer. Ensure that it is 300 ± 5 mOsm.
    4. Add AMPA receptor antagonist (NBQX) to a final concentration of 3 µM and GABA receptor antagonist (bicuculline) to a final concentration of 30 µM.
    NOTE: Neurotransmitter receptor antagonists are added to avoid recurrent network activity following neuronal stimulation[18].
    5. Warm up the physiological saline to 37 °C for use.

3. **Preparation of the Specialized High-pressure Freezer and an Automated Freeze Substitution Unit**
    1. Prior to high-pressure freezing, cool down an automated freeze substitution unit to -90 °C by filling the tank with liquid nitrogen.
    2. Cool down acetone in a small cup to -90 °C by placing it inside the specimen chamber.
    3. Fill the liquid nitrogen dewar and storage dewar of the high-pressure freezer (see the **Table of Materials**) with liquid nitrogen.
    4. Set up the light stimulation protocol using the touch screen monitor.
        1. Name the program by clicking on "Edit" next to "Program name" on the light stimulation window. Another window will pop up.
        2. Set up a program by typing "15,000 ms" in "dark phase," "100 ms" in "period," "10 ms" in "pulse," and "1" in "number of periods" for a single stimulus of 10 ms. Freeze the cells 90 ms later (**Figure 1C**).
        NOTE: The "dark phase" allows the cells to recover from light exposure during sample loading. "Period" defines the stimulation frequency. For example, if the stimulus should be applied at 20 Hz, this column should be set at 50 ms. "Pulse" defines the duration of the light stimulus. Finally, the "number of periods" defines the total number of stimuli applied. For setting up a high-frequency stimulation, please see **Figure 1E**.

    5. For a "no stimulation" control, type "15 s" in "dark phase," "0 ms" in "period," "1 ms" in "pulse," and "0" in "number of periods" in the light stimulation setup window, as described in step 2.3.4.
    NOTE: By default, the "pulse" must be at least 1 ms.
    6. On the main screen, make sure that the box for light stimulation is checked.
    7. Set up the storage protocol by clicking "Specimen Storage" on the main screen. Click on "Edit." In the following window, use "+" or "-," to select "2" to store 2 disks in each channel (3 channels in total). Check "Storage LN2 enabled."

4. **Sample Loading and Freezing in the High-pressure Freezer**
NOTE: All the sample assembly and loading steps are done under a stereomicroscope with a 7.5-60X magnification range. A tweezer is used in steps 2.4.1 - 2.4.4 to manipulate the specimens. Experiments must be performed at physiological temperature.
    1. Place one sapphire disk, cell-side facing up, in the well of the black, middle plate (**Figure 1B**).
    2. Place a 100 µm spacer ring over the sapphire disk (**Figure 1B**).
    3. Place a blank sapphire disk over the spacer ring (**Figure 1B**) after dipping one side of the disk in the pre-warmed saline solution from step 2.2. Make sure that no air bubbles are trapped between the two sapphire disks.
    4. Place another 100 µm spacer ring and a 400 µm spacer ring (**Figure 1B**). Remove the extra liquid using filter paper.
    5. Place the assembly from step 2.4.3 between the two transparent half-cylinders (**Figure 1A**). Close the top red cover to initiate the freezing process.
    NOTE: The preset protocol runs automatically once the cover is closed. The sample stays in the same orientation in the freezing chamber, and a flash of light is applied from the top of the sample assembly. The red cover pops back up automatically once the freezing process is completed.
    6. Store the specimen in the storage dewar.
    NOTE: After freezing, the specimen is automatically dropped into a storage dewar filled with liquid nitrogen and stored there until further processing. The storage dewar has three chambers, and each chamber can hold up to 3 samples at most. Typically, two specimens are frozen under the same stimulation conditions, and the high-pressure freezer is programmed to store both in the same chamber.
    7. Repeat steps 2.4.1 - 2.4.6 for each specimen.
    8. Proceed to step 2.5 once all the chambers are full.
    NOTE: The device was set to store up to 6 specimens in the storage dewar at a time. Therefore, after every 6th sample, step 2.5 must be performed. Once unloaded, repeat steps 2.4.1 - 2.4.6.

5. **Sample Collection and Transfer to an Automated Freeze-substitution Unit**
    1. Open the door to the storage dewar, which is located under the table of the high-pressure freezer. Remove the storage dewar and place it on the benchtop.
    2. Using hands, remove the specimen chamber from the dewar and transfer it in the specialized sample tray filled with liquid nitrogen. Unlock the knob to release the specimen cup.
    3. Remove the transparent half-cylinders from the specimen cup using a pair of tweezers after pre-cooling the tips of the tweezers with liquid nitrogen (~-196 °C). Carefully transfer the middle, black plate to a small cup containing liquid nitrogen.

NOTE: The tips of the tweezers must be precooled to the temperature of liquid nitrogen. The sample must be kept under liquid nitrogen at all times to prevent ice crystal formation.

3. Freeze Substitution in the Automated Freeze-substitution Unit

1. Using precooled tweezers (tips at ~-196 °C), quickly transfer the middle plate from step 2.5.3 to the precooled acetone (-90 °C).
2. Separate the sapphire disk from the middle plate by gently shaking or tapping.
   NOTE: Occasionally, it may be difficult to separate the sapphire disk from the middle plate. In such a situation, leave the middle plate in precooled acetone (-90 °C) for a few minutes. Gentle tapping with tweezers also helps to dissociate the sapphire from the middle plate.
3. Place a cryogenic vial containing fixatives (step 2.1) inside a specimen chamber of the substitution unit. Transfer the sapphire disk to the cryogenic vial and place a cap on the vial.
4. Set up the freeze substitution program as follows: (i) -90 °C for 5 - 30 h, (ii) -90 - -20 °C in 14 h (5 °C/h), (iii) -20 °C for 12 h, and (iv) -20 °C - 20 °C in 4 h (10 °C/h).
   NOTE: The duration of the first step at -90 °C could be varied. The total duration of freeze substitution is set in such a way that the program ends in the morning (~1.5 d postexperiment) around 8 am so that the subsequent steps can be performed during the daytime.

4. Infiltration and Plastic Embedding with Epoxy Resin

1. Once the program ends, use gloved hands to transfer the cryogenic vials containing the sapphire disks from the specimen chamber of the substitution unit to a chemical hood.
2. Using a pipette, add acetone (room temperature) to each cryogenic vial and wash each sapphire disk 4 - 6x for 1 - 2 h.
3. Optionally, incubate the specimens in 0.1% uranyl acetate for 1 h if extra contrast is needed. Wash 4 - 6x with acetone over 1 - 2 h.
   NOTE: CAUTION: There is risk associated with internal exposure following the inhalation of uranyl acetate, which causes irritation of the upper respiratory tract. High exposure can damage blood cells. Work with uranyl acetate should be done under exhaust ventilation. Protective clothing is recommended.
4. Prepare liquid epoxy resin medium by weighing 6.2 g of glycerol polyglycidyl ether, 4.4 g of bisphenol-A epoxy resin, and 12.2 g of dodecenyl succinic anhydride (DDSA). Mix thoroughly and add 800 µL of benzyl dimethylamine (BDMA) while mixing. De-gas for 10 min.
5. Prepare 30, 70, and 90% epoxy resin in acetone from the 100% epoxy resin prepared in step 4.4.
6. Add 30% epoxy resin to the cryogenic vials containing sapphire disks and incubate for 2 - 3 h at RT in an orbital shaker at 120 rpm.
7. Replace the 30% epoxy resin with 70% epoxy resin by pipetting and incubate for 3 - 4 h at RT in an orbital shaker.
8. Using tweezers, transfer each sapphire disk, cell-side-up, to the cap of an embedding capsule. Add 90% epoxy resin to the cap. Incubate O/N at 4 °C.
9. The next day, make fresh epoxy resin medium, as in step 4.4.
10. Transfer each sapphire disk, cell-side facing up, to the cap of an embedding capsule. Fill the cap with freshly prepared 100% epoxy resin. Change to fresh 100% epoxy resin every 2 h, repeating three times.
11. Place the samples in a 60 °C oven for 48 h to polymerize.

5. Mounting Samples

1. Place the sample upside-down on the stereomicroscope so that the sapphire disk is located at the top of the resin block. Remove the thin layer of epoxy resin from the top by scratching it with a razor blade.
2. Using a razor blade, cut a shallow line along the edge of the sapphire disk; this line helps to separate the sapphire disk from the epoxy resin in step 5.3.
3. Separate the sapphire disk from the epoxy resin by dipping it into liquid nitrogen for approximately 10 s.
   NOTE: The cells will stay in the epoxy resin.
4. After removing the sapphire disk, use a dissecting scope to find an area with cells (4-10X magnification). Cut around the region of interest (~2 x 2 mm) using a razor blade (double-edged). To keep the specimen in place, perform this step while the specimen is taped to the surface of the microscope.
5. Mount the small piece of plastic (~2 x 2 x 5 mm) containing the cells using glue containing ethyl cyanoacrylate on a cylindrical dummy block made of epoxy resin. Incubate the block at 60 °C for 1 h.

6. Sectioning

1. Use an ultramicrotome to section the sample.
   1. Trim the surface of the plastic-embedded specimen with a glass knife at a speed of 3 mm/s and a thickness of 200 nm/section. Cut 4 - 5 sections from the surface where the astrocytes are located.
   2. Switch to a diamond knife and section at a speed of 0.8 mm/s and a thickness of 40 nm/section. Cut 20 - 25 sections.
2. Collect ribbons of sections on single-slot copper grids covered with 0.7% polyvinyl acetate (see the **Table of Materials**).

7. Imaging Using a Transmission Electron Microscope (TEM)

1. Prior to TEM imaging, stain the section with 2.5% uranyl acetate in methanol for 5 min.
2. Wash the grid 15x in 50% methanol. Then, wash in ultrapure water 15x, with each wash lasting 30 s.
3. Briefly air-dry the section and place the grid into a specimen holder of the TEM.
4. Image at 93,000X magnification.

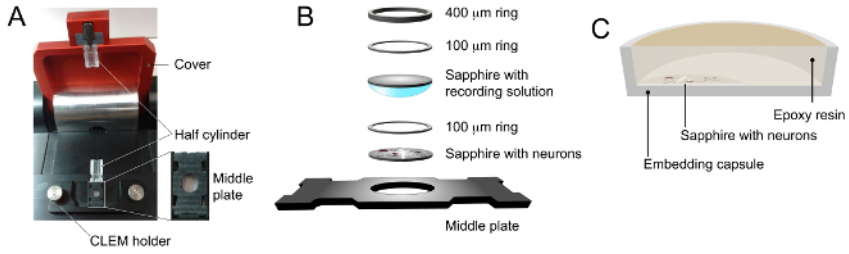
5. Acquire images.
   NOTE: Imaging is typically done blind to the time points or genotypes, and typically ~200 images are collected/time point.

8. Image Analysis

1. Analyze the images using a software (*e.g.,* ImageJ) with a custom macro (Watanabe, Davis, and Jorgensen, unpublished).
   NOTE: The x/y coordinates are recorded from vesicles, the plasma membrane, the active zone membrane, and all other membrane-bound organelles at synapses. The text files containing the information are exported to another software (*e.g.,* Matlab). The data are further analyzed using custom programs.

Representative Results

Using the protocol described above, we performed "flash-and-freeze" experiments in mouse hippocampal neurons expressing channelrhodopsin. These neurons were frozen either 15 ms or 100 ms after light onset. We have previously shown that the exocytosis and endocytosis of synaptic vesicles occur in the synaptic terminals at the 15 ms and 100 ms time points, respectively[18]. These events were successfully captured at the appropriate times (**Figure 2**), suggesting that flash-and-freeze experiments can be successfully performed on the chosen specialized high-pressure freezer (see the **Table of Materials**).



D A single stimulus, 90 ms rest

Line	Dark Phase [ms]	Period [ms]	Pulse [ms]	# Periods
1	15000	100	10	1
2	0	0	1	0

E 10 stimuli, 20 Hz, 5 ms rest

Line	Dark Phase [ms]	Period [ms]	Pulse [ms]	# Periods
1	15000	50	10	9
2	0	15	10	1

Figure 1. Sample Loading and Programming in the High-pressure Freezer. A) Sample loading table of a high-pressure freezer. The middle plate, shown in the inset for structural detail, is placed in a CLEM holder for sample loading. One of the half-cylinders is placed at the bottom part of the sample loading table, and the other is attached with a clip to the top cover. Once the sample is loaded, the middle plate is pushed forward to the bottom half-cylinder and the cover is closed to initiate the freezing. **B)** Specimen assembly. The sapphire disk containing neurons is placed in the well of the middle plate, with the cell-side facing up. A 100 µm ring is placed directly above the sapphire disk inside the well. Then, an empty sapphire disk dipped in physiological saline is placed with the solution-side down. Air bubbles must be avoided. Finally, a 100 µm ring and a 400 µm ring are snugly placed above. Any extra liquid is removed with filter paper. **C)** A cross-section of an embedding capsule with the sapphire disk submerged in epoxy resin. The sapphire disk is placed at the bottom of the capsule, with the cell-side facing up and covered with epoxy resin for infiltration and embedding. **D)** Programming the high-pressure freezer for a single, 10 ms stimulus. The specimens are frozen 90 ms after the light pulse. **E)** Programming the high-pressure freezer for 10 stimuli at 20 Hz. The specimens are frozen 5 ms after the last light pulse. Please click here to view a larger version of this figure.

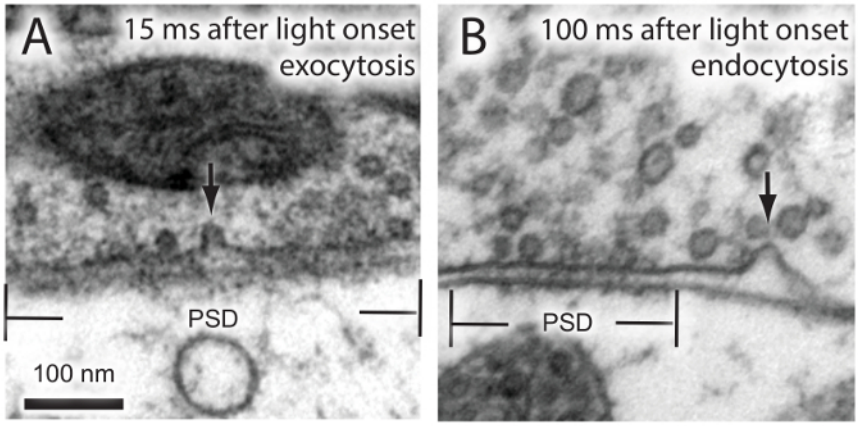


Figure 2. Visualization of Exocytosis and Endocytosis in Mouse Hippocampal Neurons. Hippocampal neurons are stimulated once and frozen at the indicated times. Electron micrographs show the exocytosis of a synaptic vesicle **A)** and ultrafast endocytosis **B)**. PSD, post-synaptic Density. Please click here to view a larger version of this figure.

Discussion

The "flash-and-freeze" approach visualizes membrane dynamics by inducing a particular cellular event with optogenetics and by freezing cells at defined time points after stimulation[19]. In this demonstration, we used channelrhodopsin, a light-sensitive cation channel, to stimulate neurons and captured the fusion and recovery of synaptic vesicles at the synaptic terminals. In recent years, many optogenetic tools have been developed[22,23], all of which are compatible with flash-and-freeze. For example, organelle trafficking can be induced using light-induced heterodimerization of cryptochrome and CIB1[24]. Similarly, the lipid composition of the plasma membrane can be altered by the light-induced translocation of phosphoinositide phosphatases to the plasma membrane[25]. Furthermore, small, light-sensitive compounds like azobenzene change conformation depending on the illumination wavelengths. This conformational change can be used to activate ligand-gated channels or to change lipid composition in the membrane[26,27]. Caged compounds can also be used to induce cellular activity. However, the LED used in the current setup may not produce sufficient energy for uncaging; thus, further optimizations of the system are likely necessary. Nevertheless, the applications of these light-activatable tools are flexible-many cellular events can be induced by a flash of light. "Flash-and-freeze" can capture the resulting membrane dynamics.

There are two main limitations to the "flash-and-freeze" method. First, it captures "snapshots" of a particular event from different cells. In other words, it is not possible to follow membrane dynamics in one cell over a period of time. Thus, for the reconstruction of any cellular event, one must acquire and analyze a large number of images from each sample and at each time point. Furthermore, in neurons, an even larger number of images is necessary, since the fusion of synaptic vesicles only takes places in 20 - 30% of the synapses in mouse hippocampal neurons[18,28]. The analysis of such a large dataset requires tremendous amounts of time. In the future, image acquisition and analysis need to be automated to make the approach more efficient[29,30].

The second limitation is imposed by the nature of the high-pressure freezing technique. When cells freeze, cellular water rearranges to form ice crystals if the freezing rate is below 100 K/s[21]. These ice crystals can penetrate membranes or concentrate solutes to alter local osmotic pressure, resulting in the rupture of membranes. To avoid ice crystals, high pressure (~2,000 atm) is applied to the specimens. Due to the super-cooling effect, a freezing rate of 100 K/s is sufficient to prevent water from forming ice crystals at this pressure[21]. In theory, specimens as thick as 500 µm can be frozen without ice crystals, but approximately 200 µm is likely the practical limit, as cuboidal forms of ice tend to form in thick tissue, compromising morphology. When processing specimens thicker than 5 µm, the use of a proper cryo-protectant, such as BSA, is necessary. However, BSA will change the osmolarity of the solution and may affect the physiological response of cells. Therefore, extensive control experiments are required to validate the use of BSA in particular systems. Ice crystals can also form after high-pressure freezing if the specimens are accidently removed from the liquid nitrogen bath. Thus, it is critical to keep the specimens in the liquid nitrogen at all times and to use pre-cooled forceps to manipulate them.

When planning experiments, the following three points should be considered. First, the maximal intensity of light (the 460 nm line) is 5.5 - 8.0 mW/mm^2. Whether this intensity is sufficient to induce activity must be verified with live-cell imaging on a fluorescence microscope prior to flash-and-freeze experiments. Second, experiments must be performed at physiological temperature. The stage of the high-pressure freezer is warmed up to 37 °C for the experiments with mouse hippocampal neurons[31]. Finally, the time points must be carefully chosen to capture the membrane dynamics. Initial studies indicated that endocytosis is complete after 100 ms of stimulation. Thus, three additional time points (15 , 30, & 50 ms) were also examined to follow the membrane dynamics[17,18]. These time points were necessary to visualize membrane trafficking events during synaptic transmission. However, the requirement for the number of time points are different in each cellular event. Therefore, a few time points should be sampled before initiating large dataset collection.

Disclosures

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