Chapter 3

Flash-and-Freeze Electron Microscopy: Coupling Optogenetics with High-Pressure Freezing

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Abstract

A complete understanding of neuronal functions requires observation of the synapse with high spatial and temporal resolution. Recently, we developed a method to combine optogenetics with electron microscopy to capture dynamic changes in synaptic morphology during neurotransmission. First we generated transgenic *C. elegans* animals expressing channelrhodopsin in specific neurons. We stimulated these neurons in intact animals using a home-built light stimulation device and modified specimen holders for the Leica EM Pact2 high-pressure freezer. Samples were subsequently frozen 20 ms after the light stimulus. We demonstrated that synaptic vesicle fusion intermediates could be captured using this technique. This method can be readily applied to other light-activatable molecules, such as caged compounds, light-switchable ligands, and photoactivatable proteins, to study dynamic changes in cells.

Key words Optogenetics, High-pressure freezing, Time-resolved electron microscopy, 4-D electron microscopy, Neurotransmission, Synaptic vesicle exocytosis

1 Introduction

Changes in membrane architecture can occur rapidly during trafficking through the secretory pathway. For example, regulated fusion at synapses is extremely fast; the synaptic delay between stimulus and postsynaptic response is only 1 ms [1]. Two problems arise for the visualization of such cellular events: size and speed. The synapse is too small to visualize membrane fusion using conventional light microscopes. Synapses in many organisms can be as small as a few hundred nanometers across, and synaptic vesicles are typically 30–40 nm in diameter; these sizes are far below the resolution limit of light microscopy. Thus, the morphology of a synapse is best imaged using electron microscopy.

Speed is the second hurdle for measuring certain cell biological processes. When neurons are stimulated, neurotransmitters are synchronously released into the synaptic cleft within a few milliseconds of the arrival of the action potential [2]. Conventional sample

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preparations for electron microscopy, unfortunately, rely on perfusion and diffusion of the fixative; these methods are too slow to capture rapid events and also introduce fixation artifacts. Worse yet, the fixatives used to prepare the samples cause the fusion of synaptic vesicles [3, 4]. Thus, capturing the very moment of vesicle fusion to the membrane seemed almost impossible. To overcome this limitation, Heuser and Reese developed the freeze slammer. In this device, specimens were slammed against a metal block cooled to 4° K by liquid helium [2]. Specimens were frozen near-instantaneously. By electrically stimulating the preparation at various time points before freezing, the temporal sequence of vesicle fusion was characterized with millisecond temporal resolution [2, 5, 6]. However, the freeze slammer was only capable of freezing the surface of the specimen without ice crystals (up to 10 μm deep), and thus only dissected preparations of neurons [2, 5, 6] or very thin tissues [7–9] could be analyzed. Moreover, the stimulation conditions required the use of potassium channel blockers and high calcium [2, 5, 6] and was thus not physiological. To apply rapid freezing methods to different model organisms, two improvements were necessary: increased vitreous freezing depth and in vivo physiological stimulation protocols.

In recent years, a high-pressure freezing method was developed to improve the freezing depth [10, 11]. Normally, when liquid water is cooled to freezing temperature, water molecules begin to form ice on a seed crystal or another nucleating structure [12]. When ice crystals form, the local concentration of solutes increases because the solutes are excluded from the crystals [12]. This increase alters the local osmotic pressure, eventually leading to the bursting of cellular membranes [12]. In the absence of a nucleation site, water can stay in its supercooled liquid phase [12] until the temperature reaches −40 °C, when it will crystallize even in the absence of a nucleus [13]. If a freezing rate of −10,000 °C/s can be achieved, supercooled water can be vitrified, that is, the water will freeze in an unordered state. Liquid nitrogen can generate a cooling rate of −16,000 °C/s. However, due to the poor heat conductance of water, the freezing rate of tissue 10 μm deep is very slow, and therefore thick samples (>10 μm) cannot be frozen without the formation of ice crystals. However, at 2,100 bar (1 bar = atmospheric pressure at sea level), water can be supercooled to −90 °C. Under these conditions, a freezing rate of −100 °C/s is sufficient to vitrify water [10]. Thus, by freezing under high pressure, biological tissues as thick as 500 μm can be frozen with reduced ice crystal formation, despite the poor heat conductance of water. Therefore, in principle, time-resolved imaging via electron microscopy can be performed in intact animals such as *Caenorhabditis elegans* using high-pressure freezing. However, neurons in intact animals are not

readily accessible by an electrode. Therefore, an alternative stimulation method must be sought.

In the last decade, optogenetic techniques have been developed to apply noninvasive stimulation to neurons and precisely control neuronal activities [14]. The discovery of light-activated sodium channels [15–17] and engineered variants such as channelrhodopsin [17–21] has made it possible to activate specific neurons in intact organisms by a simple flash of light [22–27]. Therefore, by coupling light-induced stimulation with high-pressure freezing, temporal events in neurotransmission can be studied in intact animals under physiological conditions. Unfortunately, the current configurations of commercially available high-pressure freezers do not allow for light stimulation of specimens.

We developed a device that couples optogenetics with high-pressure freezing. The device can preserve morphological changes that occur during neurotransmission with a temporal resolution of milliseconds [28, 29]. In this chapter, we describe the use of channelrhodopsin to stimulate neurons. The protocol is compatible with other methods that selectively activate or inactivate cellular activities by light, such as caged neurotransmitters [30–32], photoswitchable ligands [33, 34], caged second messengers (cyclic nucleotides) [35], and photoactivatable proteins (light-inducible protein interactions) [36–38]. For example, time-resolved electron microscopy could be used to observe the dynamic reorganization of postsynaptic terminals that occurs when synaptic facilitation is induced by caged glutamate. Cells that are naturally responsive to light such as rod or cone cells in the retina can be studied using this method. Alternatively, rapid events in non-excitable cells such as cytoskeletal reorganizations or membrane trafficking can be studied using this technique. In summary, these methods can capture cellular dynamics with nanometer spatial resolution and millisecond temporal resolution.

2 Materials

2.1 High-Pressure Freezing

1. Leica EM Pact2 high-pressure freezer with or without rapid transfer system (Leica microsystems)
2. Specimen bayonet (Leica microsystems)
3. Specimen pods (Leica microsystems)
4. Sapphire end stones (#E2.08, Swiss Jewel Company)
5. Custom-made screws
6. Two-part epoxy
7. Silver marking pen (fine point)
8. Sample cup (“membrane carriers,” 100 μm deep, #16707898, Leica microsystems)

9. Loading fork ("manual bayonet loading device," #16707828, Leica microscystems)
10. Tweezers (insulated, #16LZ01873KN, Leica microsystems)
11. Paint brush (#00)
12. Methylcyclohexane (#300306, Sigma-Aldrich)
13. Hexadecene (#H2131-100ML, Sigma-Aldrich)
14. Bovine serum albumin (A2153-10G, Sigma-Aldrich)
15. Liquid nitrogen

2.2 Light Stimulation Device

1. Modified specimen bayonet (Marine Reef International)
2. Modified specimen pods (Marine Reef International)
3. Light stimulation controller (Marine Reef International)
4. Lambda DG-4 light source (Sutter)

2.3 Freeze-Substitution

1. Automated freeze-substitution unit (AFS 2, Leica microsystem)
2. Cryovials (#D9912, Nalgene)
3. Acetone (RT10016, EMS)
4. Ethanol (#459844-1L, Sigma-Aldrich)
5. Osmium tetroxide (crystals, 1/10 g; RT19134, EMS)
6. Glutaraldehyde in acetone (#16530, EMS)
7. Uranyl acetate (#21447-252, Polysciences)
8. Disposable transfer pipette (#14670-201, VWR)
9. Disposable Pasteur pipette (Borosilicate glass; #13-678-20A, Fisher)

2.4 Plastic Embedding

1. Epon-araldite (kit, #18028, Ted Pella)
2. BEEM capsule (#70010B, EMS)

2.5 Sectioning

1. Ultramicrotome (UC6, Leica microsystem)
2. Diamond knife (Ultra jumbo, 45°, 4.0 mm; DiATOME)
3. Glass strips (#8030, Ted Pella)
4. Glass knife boats (#123-3, Ted Pella)
5. Hair tool for manipulation of plastic sections
6. Razor blade (Double edge; #72000, EMS)
7. High profile microtome blades (#818, Leica microsystem)
8. TEM grids (single slot, #1GC12H, Ted Pella)
9. Formvar (0.5 %, RT15820, EMS)

2.6 Imaging

1. Transmission electron microscope
2. Digital camera (Orius, Gatan)

3 Methods

3.1 Modification of Specimen Holders

A high-pressure freezer is capable of freezing a thick specimen with reduced ice crystal formation. However, specimens must be mounted within a closed compartment and cannot be illuminated (Fig. 1a, b). The Leica EM Pact2 was developed to allow direct observation of specimens under a light microscope up to a few seconds before freezing [39–41]. Specifically, specimens are loaded into an open cup and capped with a black diamond on a specimen pod (Fig. 1b–d) just prior to freezing either manually or via a rapid transfer system (RTS). The transfer system screws down the black diamond anvil to cover the sample cup and drives the specimen bayonet into the freezing chamber automatically. The transfer system can be used to capture mem-

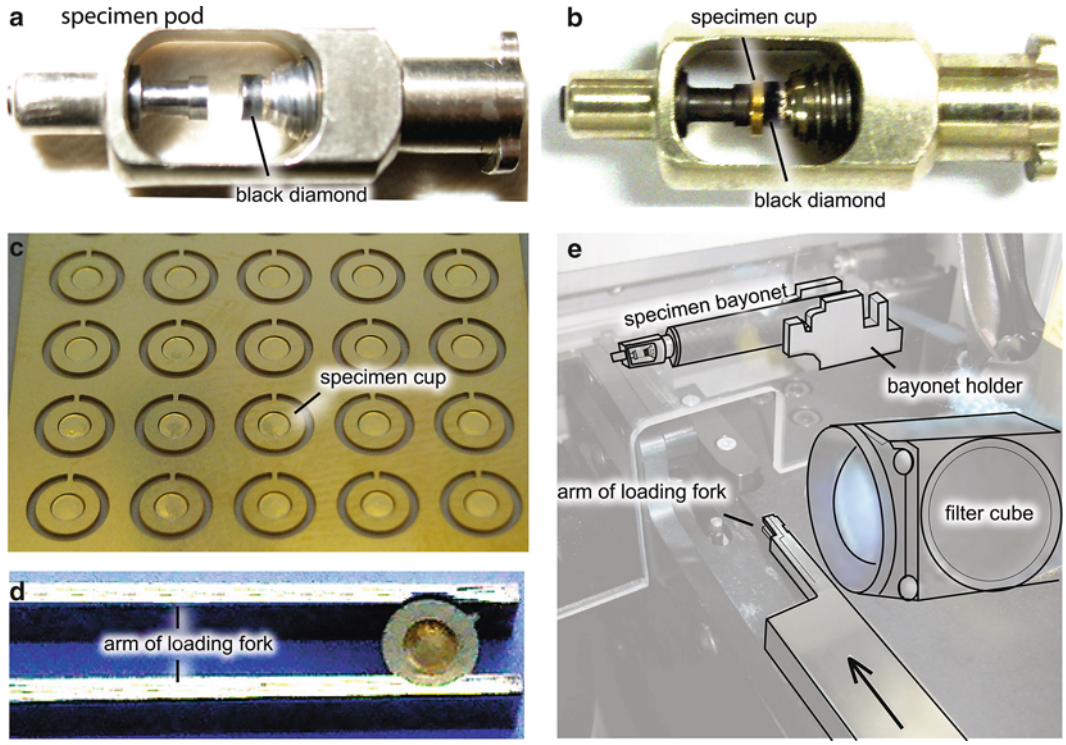

Fig. 1 Specimen mounting. (**a**) A specimen pod without the sample cup. (**b**) A specimen pod with a sample cup in place. Note that once the carrier is mounted in the pod, the specimens face the *black diamond* and cannot be exposed to light. (**c**) Sheet of 20 sample cups for the EM Pact2. Each cup can be detached from the surrounding metal by prying back and forth with forceps. Specimens are mounted into the depression of the sample cup (1.6 mm in diameter and 100 μm deep). (**d**) A sample cup is loaded into the arm of the loading fork. Note that the specimens are in an open cup and could potentially be exposed to light. (**e**) The experimental setup for light stimulation using the EM Pact2-RTS. A filter cube attached at the tip of an optical fiber can be placed next to the specimen loading path to shine light onto the specimen for up to two seconds before freezing. After stimulation, specimens are screwed into the specimen pod simply by sliding the loading device to the specimen pod (indicated by a *black arrow* on the loading device). Once the sample cup is screwed securely into the pod, the specimen bayonet is rapidly driven down a rail into the freezing chamber

brane dynamics at synapses ~2.5 s or greater after chronic or repetitive stimulation. An optical fiber with a filter cube can be mounted adjacent to the specimen loading path (Fig. 1e). Light stimulation can be applied either from a mercury lamp or a laser source before freezing the sample. However, to resolve the membrane dynamics involved in processes such as exocytosis and rapid forms of endocytosis requires millisecond temporal resolution [2, 5, 6, 42]. To improve the temporal resolution, we built a device and computer program to stimulate samples milliseconds before freezing. In this chapter, we describe in detail how to build and operate this device.

First, we modified the specimen bayonet so that an LED bulb or an optical fiber could be mounted adjacent to the sample cup. To create this light path to the specimen, three modifications were required (Fig. 2). First, a hole was drilled into a specimen bayonet. A specimen bayonet (Fig. 2a) has 6 components: an insulating cover, a metal shaft, a metal chuck, a spring, a pin, and a cap (Fig. 2b). The metal chuck and spring are used to lock the specimen pod tightly at the tip of the bayonet. The metal shaft can be removed from the insulating cover, freeing the metal chuck and spring from the holder. The pin was removed and a 2 mm hole drilled through the middle of the metal shaft to create a conduit for wires (Fig. 2c). Second, we replaced the metal chuck with a 3.2 mm LED bulb and a fabricated metal LED mount (Fig. 2d). Third, we fabricated a new screw for a specimen pod (Fig. 2e). The original screw has a black diamond anvil attached at the tip (Fig. 2e left). The fabricated screw has a bore with a diameter of 1.2 mm down the central axis and a countersink with a diameter of 2.08 mm at the tip of the screw (Fig. 2e right; *see* **Note 1**). A transparent sapphire anvil with a diameter of 2.08 mm and a thickness of ~1 mm was glued in the countersink using two-part epoxy (*see* **Note 2**). The modified specimen bayonet and pod were reassembled to the original configuration. The new specimen holder allows for the application of single-color light with an intensity as high as ~20 mW/mm^2.

An alternative assembly allows the application of light of multiple wavelengths. The bayonet in this configuration holds an optical fiber. In addition to the modifications described above, the pin was split into two halves and glued back into the shaft with two-part epoxy, leaving the central bore open for the optical fiber. An optical fiber with a diameter of 1.5 mm was placed in the central bore of the shaft, and the free end of the fiber was attached to a Lambda DG-4 light source. To house the optical fiber in the freezing path of the rapid transfer system, we had to remove the safety cover (Fig. 3). Because the safety lock was controlled by magnets attached to the cover, we removed the magnets from the cover and placed them directly on the sensor (Figs. 3 and 4a; see **Note 3**). These modifications allowed the application of multiple colors of light prior to freezing. However, we have not yet developed a computer interface to the Lambda DG-4 for high temporal resolution control.

Fig. 2 Modifications of specimen bayonet and pod (**a**) the original specimen bayonet and pod. (**b**) All six components of the specimen bayonet. (**c**) Schematic diagrams for bayonet modifications. The *dotted lines* represent the holes in the bayonet. We drilled a 2 mm hole in the middle as indicated in the modified shaft. We placed an LED onto the modified chuck. Finally, the modified pieces were assembled. (**d**) A modified chuck without (*left*) and with an LED (*right*). (**e**) Schematic diagrams (*top*) and photographs (*bottom*) showing the modified specimen pod. A screw with a 1.2 mm hole (*dotted lines*) was fabricated, and a sapphire anvil was glued into the countersink. These modifications allow for the application of light to the specimens milliseconds before freezing

3.2 Development of the Light Stimulation Device

To observe membrane dynamics at synapses, light stimulation must be applied to specimens with millisecond temporal precision. For the EM Pact2 with a Rapid Transfer System attachment, a specimen bayonet is driven into the freezing chamber by pressing the start button on the LED display. The freezing process is initiated when the specimen bayonet is securely locked into position.

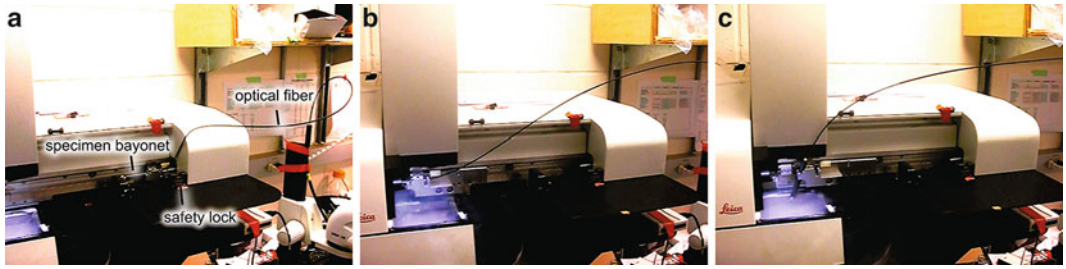

Fig. 3 Optional fiber-optic configuration. (**a**) An optical fiber can be mounted into the modified bayonet. (**b**) A sample holder is driven into the freezing chamber. (**c**) The specimen bayonet is retracted from the freezing chamber, and the sample drops into the liquid nitrogen bath following high-pressure freezing. Note that the angle of the specimen bayonet in (**c**) is tilted slightly by the fiber-optic cable and should be righted manually so that the specimen is fully immersed in the liquid nitrogen

For an EM Pact2 without the transfer attachment, a specimen bayonet is loaded directly into the freezing chamber. Pressing the start button will then initiate the freezing process. To achieve millisecond temporal resolution, the interval between when the start button is pressed and when the specimen is actually frozen must be determined. The manufacturer estimated this interval to be about 2.5 s for an EM Pact2 outfitted with the transfer system. Taking this estimate into account is adequate to achieve sub-second precision, but we sought an alternative method to achieve millisecond temporal precision.

To achieve higher temporal precision, we used an accelerometer to determine when the sample is fired down the rail, when it enters the freezing chamber, and when it is pressurized. A computer then controls when the light is switched on relative to these events. We installed an accelerometer on the bayonet holder on the transfer device (Fig. 4a). Interestingly, the readout of the accelerometer showed several intervals with multiple peaks indicating different motions of the specimen bayonet (Fig. 4b). The first 70 ms of the recording (interval 1) reflects the deceleration of the specimen bayonet along the rail (the recording begins after the sample was fired down the rail). To reveal what the other intervals represent, we monitored the temperature change using a thermistor while videotaping the freezer. The response time of the thermistor is too slow to capture the rapid freezing rate, but we can approximate the time when freezing occurs based on the initial response recorded by the thermistor. We found that freezing was initiated roughly 200 ms after the specimen bayonet reached the freezing chamber. This suggests that the first downward deflection observed in interval 3 reflects when pressure was applied to the specimen since this peak appears nearly simultaneously with freezing (Fig. 4b). The pressure application, thus, occurred 1,545 ms after the accelerometer detected the initial launch of the bayonet down the rail (*see* **Note 4**).

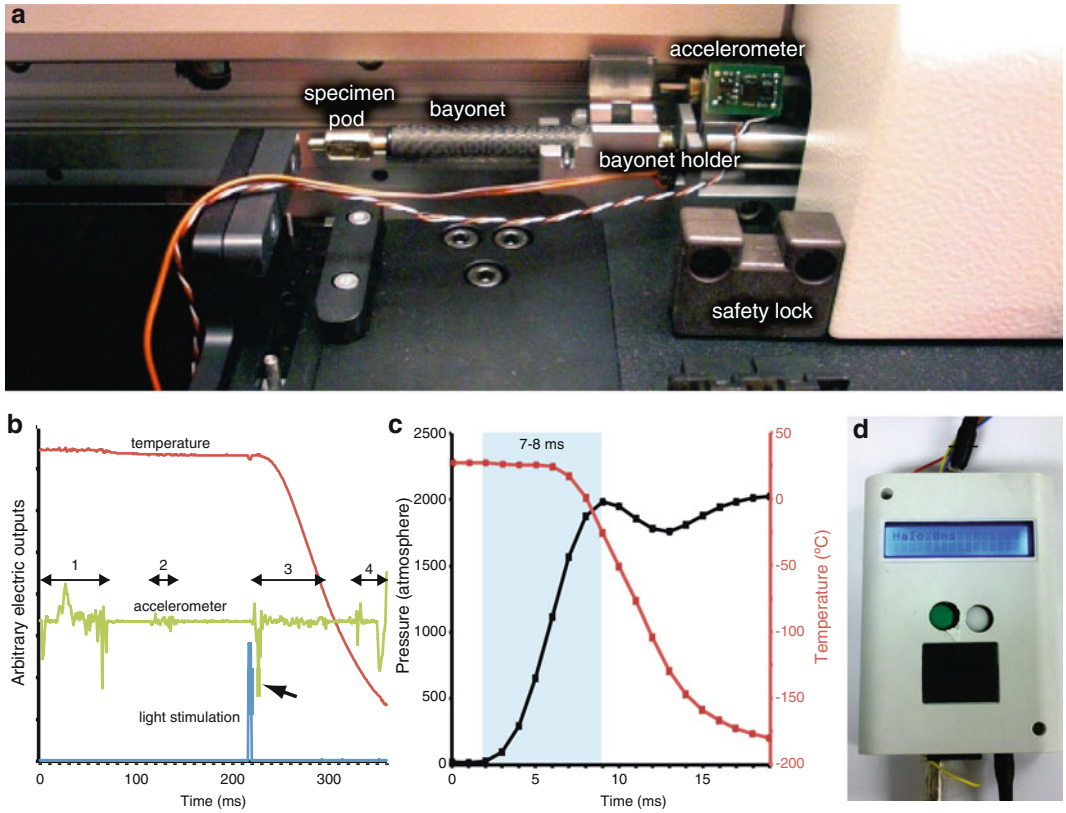

Fig. 4 Measuring the light flash to freezing interval. Temporal resolution for light stimulation is improved by implementing an accelerometer. (**a**) An accelerometer is attached to the bayonet holder. (**b**) A graph showing the readouts from a light stimulation device (*blue*), a thermistor (*red*), and an accelerometer (*green*). The outputs from the sensors were all recorded by a computer program. The readout from the accelerometer shows four intervals with multiple peaks. Interval 1 indicates the termination of the specimen bayonet movement along the rail as it enters the freezing chamber. The specimen bayonet is locked in place for freezing during interval 2. Pressure application occurs at the first downward deflection indicated by a *black arrow* in the interval 3. The specimen bayonet is retracted from the chamber during interval 4. (**c**) A graph showing the pressure and temperature readouts recorded by the high-pressure freezer. Pressure is indicated by the *black line*, and its value is found on the primary *y*-axis (*black line*). Temperature is indicated by the *red line*, and its value is found on the secondary *y*-axis (*red line*). There is a delay of about 7–8 ms from the application of pressure to the freezing of specimens below 0°C. (**d**) The final light stimulation controller. The controller sends out signals to fire both the light stimulation and the freezer upon pressing the *green button*. The light stimulation protocols can be programmed using a computer, sent to the controller via a USB cable, and stored in a microSD card inserted into the controller. Different stimulation protocols can be selected by pressing the *white button*. The display shows the current protocol selected

Telemetry from the high-pressure freezer further refines the interval between the application of pressure and freezing at a higher temporal resolution. This interval was consistently 7–8 ms from shot to shot (Fig. 4c; *see* **Note 5**). Therefore, about 1,555 ms is required to freeze the specimens after pressing the start button and launching the bayonet. These results suggest that if a light pulse of

20 ms is to be delivered 100 ms before freezing, light stimulation should be triggered 1,435 ms after the initial reading from the accelerometer. Unfortunately, the time when the specimens are frozen varied from shot to shot by about 20 ms, so the light stimulation was not delivered consistently at the programmed time point (*see* **Note 4**). Nevertheless, we can calculate the actual interval between stimulation and freezing based on the accelerometer readings from each shot.

The Leica EM Pact2 can also be operated without the transfer system. In this configuration, the specimen bayonet is manually locked in the freezing chamber, and thus the accelerometer readout cannot be used to deliver the light stimulation at the desired time point. We consulted Leica technicians and obtained an interface to the computer in the instrument. We built an electronic light stimulation controller (Fig. 4d) to send a "start" signal for freezing, bypassing the necessity to press the start button on the display. The control box can apply light stimulation with sub-10 ms temporal resolution. Nevertheless, there is still a variable delay introduced by the freezer that must be accounted for (*see* **Note 4**). The light stimulation controller and the customized specimen bayonet and pods are now commercially available from Marine Reef International (*see* **Note 6**).

3.3 Light Stimulation-Coupled High-Pressure Freezing

For light stimulation-coupled high-pressure freezing, specimens can be mounted into a sample cup using procedures appropriate for each model system. Procedures for mounting particular specimens have been described extensively [39, 43–45]. For *C. elegans*, animals expressing channelrhodopsin need to be placed on a plate containing trans-retinal at least 16 h prior to the experiments [25]. Once animals are transferred to a plate containing trans-retinal, they should be kept in darkness. The high-pressure freezing should also be performed in a dim room to minimize light exposure. To freeze *C. elegans* animals, a 100 μm deep sample cup should be used. Bacteria (*E. coli*) or 20 % BSA can be used to fill the cup and to act as a cryoprotectant (*see* **Note 7**) [39]. We use a manual loading station to mount the specimen carrier into the specimen pod. Because specimens are inevitably exposed to light while mounting, once specimens are loaded into the pod, the pod should be placed in the dark for at least 30 s to allow recovery. For freezers lacking the transfer system, the pod can be mounted onto the bayonet and directly inserted into the freezing chamber for this step. In the meantime, the stimulation protocol should be programmed and selected using the light stimulation controller (*see* **Note 8**). Once the specimen bayonet is locked, light stimulation and freezing can be triggered by simply pressing the green button on the light stimulation device controller (Fig. 4d). Between each shot, testing the function of LED may be necessary, since freezing may damage the wiring or the LED itself. The frozen specimens can be processed using standard procedures for freeze-substitution and plastic embedding [39, 43, 44, 46, 47].

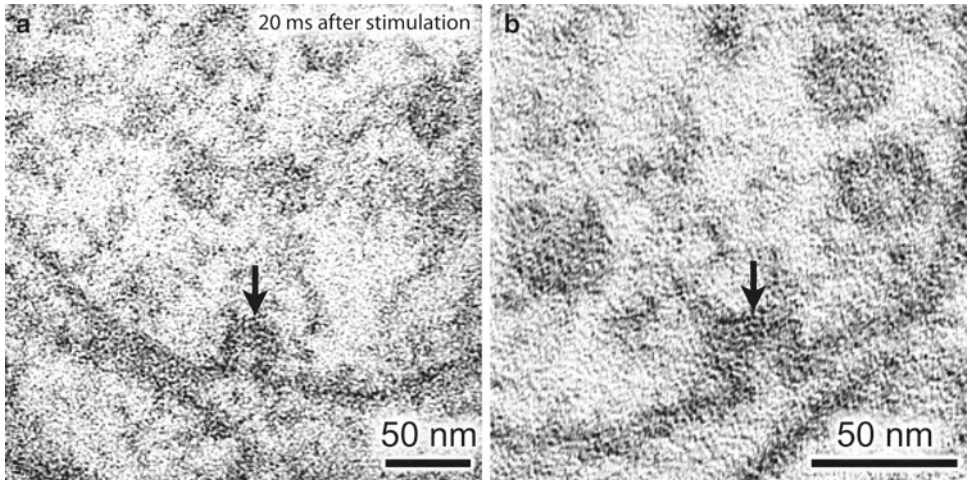

Fig. 5 Fusion intermediates are captured by freezing specimens 20 ms after light stimulation. (**a**–**b**) Electron micrographs of *C. elegans* motor neurons showing synaptic vesicle fusion intermediates. A single light pulse of 20 ms was applied to transgenic animals expressing ChIEF 20 ms before high-pressure freezing

4 Application Examples

Using the light stimulation device, we monitored membrane dynamics in *C. elegans* neurons expressing ChIEF [21], a variant of channelrhodopsin. We programmed our light stimulation device to fire a single 20 ms light pulse 20 ms before freezing. At 20 ms, vesicles can be observed collapsing into the membrane [5] along the active zone of motor neurons (Fig. 5). This result suggested that a combination of high-pressure freezing and optogenetics is capable of capturing membrane dynamics with high temporal resolution.

5 Notes

1. The countersink of the screw must be fabricated precisely so that the surface is absolutely flat. An uneven surface will cause the sapphire anvil to be slightly tilted, resulting in a pressure leak from the specimen carrier while freezing. In addition, tilted sapphire anvils tend to shatter when subjected to high pressure.
2. The amount of two-part epoxy applied to the rim of the countersink should be minimal—just enough to cover the rim. Otherwise, the bottom surface of the sapphire that faces the light source will have a thick layer of glue, resulting in the reduction in the transmitted light intensity. A silver marking pen can be used to coat the side of the sapphire anvil with a reflective surface to maximize the light intensity transmitted to the specimen.

3. Because safety features are bypassed, you are responsible for any injury that may occur. Make sure you do not stick any part of your body near the moving parts if you are using an EM Pact2 with a Rapid Transfer System. Before the machine initiates freezing, it checks to determine that the specimen is loaded and the cover is closed. Therefore, although the specimen can be mounted onto the freezing device without opening the cover, the instrument needs to receive instructions that the cover has been opened and closed. Therefore, the magnet should be removed once, indicating the opening of the cover, and placed back into position, indicating the closing of the cover. The freezing can then be triggered by pressing the start button. Finally, the optical fiber is heavy, and thus the specimen bayonet is not balanced on the bayonet holder. When the specimen bayonet comes out of the freezing chamber after freezing, the specimen pod may not fully rotate into the liquid nitrogen bath. We typically push the pod into the bath manually, but extra caution should be taken to stand away from the bayonet until it has been ejected from the freezing chamber because the chamber is under a great deal of pressure.
4. The interval between pressing the start button and freezing is ~1,555 ms for the RTS EM Pact2 and ~180 ms for the non-RTS EM Pact2. However, these values vary by about 20 ms from shot to shot. These fluctuations are likely caused by the mechanism of the freezer: Before liquid nitrogen is applied to the specimens, the freezer must ensure that the pressure in the freezing chamber is correct. The pressure is dependent on multiple factors such as the amount of liquid nitrogen in the storage tank, the idling time between shots, and the working pressure of the compressor. Thus, the light stimulation can be triggered very close to the programmed time point, but it often varies by 20 ms. Therefore, we always calculate the time when the light stimulation was actually delivered in relationship to pressurization and freezing based on the accelerometer reading for the pressure application (the lowest peak indicated by a black arrow in interval 3 in Fig. 4b).
5. The data files can be exported as .DGM format from the high-pressure freezer using a USB drive provided by the manufacturer. The file can then be converted into .CSV format using the software provided on the USB drive. By plotting the pressure data and the temperature data in Microsoft Excel, we calculated the average time required to freeze specimens to 0 °C from the time the pressure begins to increase. The average time was 8 ms ± 1 ms from 20 different freezes. This represents the shortest time interval between stimulation and freezing using this apparatus.
6. For a commercial version of light stimulation kits, contact Marine Reef International (sales@marinereef.com).

7. To maximize the exposure of the animals to the light, 20 % BSA is preferable. However, specimens frozen with 20 % BSA tend to stay stuck in the sample cup during the freeze-substitution and plastic embedding in BSA. Using force to retrieve the specimens from the carrier often results in the permanent damage to the tissues or, in the worst case scenario, permanent loss of the tissues. Thus, although some light may be scattered by bacteria during the stimulation, bacteria are better suited as space fillers for light-coupled high-pressure freezing. Heat is conducted faster in bacteria than air, and thus the bacteria serves as a cryoprotectant.
8. The programming of light stimulation protocols is very flexible. For example, a single stimulus lasting 5 ms can be applied 20 ms before the freezing (*see* **Note 4**). A train of stimuli can also be programmed (e.g., 10 Hz train for 30 s with the duration of each stimulus of 20 ms). Intervals between two trains of stimuli can also be programmed (e.g., 10 Hz for 30 s, 5 s rest, and a single stimulus of 20 ms). These programs can be stored directly in the light stimulation controller and can be selected by pressing the white button (Fig. 4d).

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References

1. Eccles SJC (1964) The physiology of synapses. Springer, Berlin
2. Heuser JE, Reese TS, Dennis MJ, Jan Y, Jan L, Evans L (1979) Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. J Cell Biol 81(2): 275–300
3. Smith JE, Reese TS (1980) Use of aldehyde fixatives to determine the rate of synaptic transmitter release. J Exp Biol 89:19–29
4. Rosenmund C, Stevens CF (1997) The rate of aldehyde fixation of the exocytotic machinery in cultured hippocampal synapses. J Neurosci Methods 76(1):1–5
5. Heuser JE, Reese TS (1981) Structural changes after transmitter release at the frog neuromuscular junction. J Cell Biol 88(3):564–580
6. Torri-Tarelli F, Grohovaz F, Fesce R, Ceccarelli B (1985) Temporal coincidence between synaptic vesicle fusion and quantal secretion of acetylcholine. J Cell Biol 101(4):1386–1399
7. Kachar B, Christakis NA, Reese TS, Lane NJ (1986) The intramembrane structure of septate junctions based on direct freezing. J Cell Sci 80:13–28
8. Arsenault AL, Ottensmeyer FP, Heath IB (1988) An electron microscopic and spectroscopic study of murine epiphyseal cartilage: analysis of fine

structure and matrix vesicles preserved by slam freezing and freeze substitution. J Ultrastruct Mol Struct Res 98(1):32–47
9. Neill PJ, Smith JH, Doughty BL, Kemp M (1988) The ultrastructure of the Schistosoma mansoni egg. Am J Trop Med Hyg 39(1): 52–65
10. Moor H (1987) Theory and practice of high pressure freezing. Cryotechniques in Biological Electron Microscopy. Springer, Berlin, pp 175–191
11. Moor H, Riehle U (1968) Snap-freezing under high pressure: a new fixation technique for freeze-etching. Proc Fourth Eur Reg Conf Electron Microsc 2:33–34
12. Dubochet J (2007) The physics of rapid cooling and its implications for cryoimmobilization of cells. Cell Electron Microsc 79:7–21
13. Rasmussen DH, Macaulay MN, MacKenzie AP (1975) Supercooling and nucleation of ice in single cells. Cryobiology 12(4):328–339
14. Yizhar O, Fenno LE, Davidson TJ, Mogri M, Deisseroth K (2011) Optogenetics in neural systems. Neuron 71(1):9–34
15. Sineshchekov OA, Jung K-H, Spudich JL (2002) Two rhodopsins mediate phototaxis to low- and high-intensity light in Chlamydomonas reinhardtii. Proc Natl Acad Sci U S A 99(13):8689–8694
16. Nagel G, Ollig D, Fuhrmann M, Kateriya S, Musti AM, Bamberg E, Hegemann P (2002) Channelrhodopsin-1: a light-gated proton channel in green algae. Science 296(5577): 2395–2398
17. Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, Ollig D, Hegemann P, Bamberg E (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci U S A 100(24):13940–13945
18. Govorunova EG, Spudich EN, Lane CE, Sineshchekov OA, Spudich JL (2011) New channelrhodopsin with a red-shifted spectrum and rapid kinetics from Mesostigma viride. MBio 2(3):e00115–00111
19. Gunaydin LA, Yizhar O, Berndt A, Sohal VS, Deisseroth K, Hegemann P (2010) Ultrafast optogenetic control. Nat Neurosci 13(3): 387–392
20. Lin JY (2012) Optogenetic excitation of neurons with channelrhodopsins: light instrumentation, expression systems, and channelrhodopsin variants. Prog Brain Res 196:29–47
21. Lin JY, Lin MZ, Steinbach P, Tsien RY (2009) Characterization of engineered channelrhodopsin variants with improved properties and kinetics. Biophys J 96(5):1803–1814
22. Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K (2005) Millisecond-timescale, genetically targeted optical control of neural activity. Nat Neurosci 8(9):1263–1268
23. Douglass AD, Kraves S, Deisseroth K, Schier AF, Engert F (2008) Escape behavior elicited by single, channelrhodopsin-2-evoked spikes in zebrafish somatosensory neurons. Curr Biol 18(15):1133–1137
24. Leifer AM, Fang-Yen C, Gershow M, Alkema MJ, Samuel ADT (2011) Optogenetic manipulation of neural activity in freely moving Caenorhabditis elegans. Nat Methods 8(2): 147–152
25. Liu Q, Hollopeter G, Jorgensen EM (2009) Graded synaptic transmission at the Caenorhabditis elegans neuromuscular junction. Proc Natl Acad Sci 106(26):10823–10828
26. Liewald JF, Brauner M, Stephens GJ, Bouhours M, Schultheis C, Zhen M, Gottschalk A (2008) Optogenetic analysis of synaptic function. Nat Methods 5(10):895–902
27. Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, Gottschalk A (2005) Light activation of channelrhodopsin-2 in excitable cells of Caenorhabditis elegans triggers rapid behavioral responses. Curr Biol 15(24):2279–2284
28. Watanabe S, Rost BR, Camacho-Perez M, Davis MW, Söhl-Kielczynski B, Rosenmund C, Jorgesen EM. Ultrafast endocytosis at mouse hippocampal synapses. Nature (advance online publication)
29. Watanabe S, Liu Q, Davis MW, Hollopeter G., Thomas N, Jorgensen NB, Jorgensen EM (2013) Ultrafast endocytosis at Caenorhabditis elegans neuromuscular junctions. Elife 2, e00723
30. Milburn T, Matsubara N, Billington AP, Udgaonkar JB, Walker JW, Carpenter BK, Webb WW, Marque J, Denk W, McCray JA (1989) Synthesis, photochemistry, and biological activity of a caged photolabile acetylcholine receptor ligand. Biochemistry 28(1):49–55
31. Walker JW, McCray JA, Hess GP (1986) Photolabile protecting groups for an acetylcholine receptor ligand. Synthesis and photochemistry of a new class of o-nitrobenzyl derivatives and their effects on receptor function. Biochemistry 25(7):1799–1805
32. Wieboldt R, Gee KR, Niu L, Ramesh D, Carpenter BK, Hess GP (1994) Photolabile precursors of glutamate: synthesis, photochemical properties, and activation of glutamate receptors on a microsecond time scale. Proc Natl Acad Sci U S A 91(19):8752–8756
33. Banghart M, Borges K, Isacoff E, Trauner D, Kramer RH (2004) Light-activated ion channels for remote control of neuronal firing. Nat Neurosci 7(12):1381–1386
34. Bartels E, Wassermann NH, Erlanger BF (1971) Photochromic activators of the acetylcholine

receptor. Proc Natl Acad Sci U S A 68(8):1820–1823
35. Adams SR, Tsien RY (1993) Controlling cell chemistry with caged compounds. Annu Rev Physiol 55(1):755–784
36. Kennedy MJ, Hughes RM, Peteya LA, Schwartz JW, Ehlers MD, Tucker CL (2010) Rapid blue-light-mediated induction of protein interactions in living cells. Nat Methods 7(12):973–975
37. Levskaya A, Weiner OD, Lim WA, Voigt CA (2009) Spatiotemporal control of cell signalling using a light-switchable protein interaction. Nature 461(7266):997–1001
38. Yazawa M, Sadaghiani AM, Hsueh B, Dolmetsch RE (2009) Induction of protein-protein interactions in live cells using light. Nat Biotechnol 27(10):941–945
39. McDonald KL, Morphew M, Verkade P, Müller-Reichert T (2007) Recent advances in high-pressure freezing: equipment- and specimen-loading methods. Methods Mol Biol 369:143–173
40. Müller-Reichert T, Mäntler J, Srayko M, O'Toole E (2008) Electron microscopy of the early Caenorhabditis elegans embryo. J Microsc 230(2):297–307
41. Verkade P (2008) Moving EM: the Rapid Transfer System as a new tool for correlative light and electron microscopy and high throughput for high-pressure freezing. J Microsc 230(Pt 2):317–328
42. Miller TM, Heuser JE (1984) Endocytosis of synaptic vesicle membrane at the frog neuromuscular junction. J Cell Biol 98(2): 685–698
43. McDonald K, Schwarz H, Müller-Reichert T, Webb R, Buser C, Morphew M (2010) "Tips and tricks" for high-pressure freezing of model systems. Methods Cell Biol 96:671–693
44. Mueller-Reichert T (2010) Electron microscopy of model systems, vol 96, 1st edn. Academic, New York
45. McDonald K (2007) Cryopreparation methods for electron microscopy of selected model systems. Methods Cell Biol 79:23–56
46. McDonald KL, Webb RI (2011) Freeze substitution in 3 hours or less. J Microsc 243(3):227–233
47. Rostaing P, Weimer RM, Jorgensen EM, Triller A, Bessereau J-L (2004) Preservation of immunoreactivity and fine structure of adult C. elegans tissues using high-pressure freezing. J Histochem Cytochem 52(1):1–12