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Calcium Imaging of Neuronal Circuits In Vivo Using a Circuit-Tracing Pseudorabies Virus

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INTRODUCTION

Pseudorabies virus (PRV) is a neuroinvasive virus of the herpes family that has a broad host range but does not infect higher-order primates. PRV characteristically travels along chains of synaptically connected neurons and has been used extensively for elucidating neural circuits in the peripheral and central nervous system in vivo. The recombinant virus PRV369 is an attenuated retrograde tracer that encodes G-CaMP2, a fluorescent calcium sensor protein that is stable at physiological pH and mammalian temperature. This protocol describes the use of PRV369 to express G-CaMP2 in a neuronal circuit and to monitor its activity in a living animal, specifically in the submandibular ganglia (SMG), the peripheral parasympathetic ganglia that innervate the salivary glands. The procedure describes the delivery of PRV369 to the glands and shows how SMG neurons can then be imaged post-inoculation to explore connectivity and activity.

RELATED INFORMATION

Additional information on the use of PRVs for tracing neural circuitry in vivo is described in Song et al. (2005). Specific information on the recombinant PRV369 is available in Granstedt et al. (2009). Background on the properties and uses of G-CaMP2 is also available (Nakai et al. 2001; Ohkura et al. 2005; Tallini et al. 2006; Hoogland et al. 2009).

PRV has a broad host range, infecting almost all mammals and some avian embryos (except higher primates including humans). Therefore, this technology is expected to work in any species susceptible to PRV infection.

For an example of an SMG infected by PRV369 and spontaneous calcium transients imaged with two-photon microscopy, see Figure 1.

MATERIALS

Reagents

Buprenorphine (0.3 mg/mL; e.g., Buprenex)

Dilute the stock 20-fold with 0.9% saline to a final concentration of 15 µg/mL

Disinfectant solution/surgical scrub (e.g., Betadine)

Use a solution with both antiseptic microbicidal and sudsing properties.

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Ethanol (70%)

Isoflurane

Ketamine/xylazine hydrochloride solution (80 mg/mL and 12 mg/mL, respectively; Sigma K113)

Dilute 10-fold with 0.9% saline to a final concentration of 8 mg/mL ketamine and 1.2 mg/mL xylazine.

Mammalian Ringer's solution (prewarmed)

Mice

If another species is used, surgical conditions and drug dosages should be optimized for that species. All procedures must be approved by local animal use and welfare authorities.

Phosphate-buffered saline (e.g., DPBS; GIBCO/Invitrogen)

PRV369 inoculum (10^{10} plaque-forming units [pfu]/mL)

*The inoculum can be obtained from the **Enquist** laboratory, Princeton University.*

Equipment

Alcohol swabs

Anesthesia chamber (plastic, equipped with nose mask and filter)

Applicators (cotton-tipped)

Cardboard

Forceps (Adson, with teeth)

Forceps (fine, #5)

Forceps (serrated, standard pattern)

Hamilton syringe (10- μ L, equipped with 33-gauge needle [0.5-in., 20° bevel])

Isoflurane vaporizer (equipped with O₂ tank and nose cone)

Microscope (dissection; e.g., Leica MZ16)

Microscope (equipped for confocal or two-photon imaging, and with a small metal platform controlled by a micromanipulator; adapted from Purves and Lichtman [1987])

Needle holders (Roboz RS-7842)

Perfusion tubes

Razor blades

Retractors

Rodent blanket (homeothermic, equipped with rectal probe and temperature controller; e.g., Stoelting Co. 50300)

Rubber band

Scalpel handle and blade

Scissors

Scissors (stitch; Roboz RS-5950)

Scissors (tissue-separator; Fine Science Tools 14072-10)

Sutures (silk, 6-0; e.g., Moore Medical 54114)

Syringe and needle for intraperitoneal injection

Tape

METHOD

Injection of PRV369 into Salivary Glands

The procedure must be performed under Biosafety Level 2 conditions.

- 1 Anesthetize the animal by injecting intraperitoneally with 16 μ L ketamine/xylazine per gram of body weight.
Wait until the animal is anesthetized by checking paw-pinch withdrawal and corneal reflexes before continuing.
- 2 Lay the animal on its back on a piece of cardboard. Immobilize the limbs with tape. Stretch back the nose by securing a rubber band across the mouth.
Monitor the animal during the entire surgical procedure and test the depth of anesthesia regularly (e.g., by controlling respiratory rate and paw-pinch withdrawal reflex).
- 3 Using an alcohol swab, wet the neck area from the base of the chin to just above the ribcage.
- 4 Use a razor blade to shave away the hair, exposing the skin.
- 5 Use cotton-tipped applicators to apply disinfectant solution/surgical scrub to the shaved area. Finish by swabbing with 70% ethanol.
- 6 Grasp the skin with forceps. Use a sterile scalpel to make a shallow incision ~1.5-cm long from the ribcage to the chin.
- 7 Gently detach the glands from the skin using tissue separator scissors.
The salivary glands are located immediately below the skin, and adhere to it via layers of connective tissue.
- 8 Rinse the Hamilton syringe with DPBS before drawing up PRV inoculum.
- 9 Using the Hamilton syringe, inject 5 μ L of the PRV369 into each side of the salivary glands:
 - i. Inject PRV into one side of the salivary gland by making two to three separate injections in the center.

- ii. Keep the syringe in place for ~10 sec at each injection site.
- iii. Repeat with the other side.

Injecting in both the left and right glands doubles the chance of finding infected cells and reduces the number of animals used.

- 10 Close the incision with silk sutures. Apply disinfectant solution to the sutured area.
- 11 For post-operative analgesia, inject 5 μ L of the diluted buprenorphine per gram of body weight intraperitoneally.
- 12 Keep the mouse on a heating blanket at 37°C until it recovers. Put the animal in a new cage by itself.

In Vivo Imaging of Fluorescent Indicator in SMG Neurons

- 13 At the desired time post-inoculation, anesthetize the animal for ~10 min in an isoflurane chamber using 2% isoflurane.
- 14 Place the animal on its back on the imaging stage. Position the animal's nose in the isoflurane cone. Tape down all limbs.
- 15 Insert the rectal temperature probe. Turn on the heating blanket to keep the animal's temperature at 37°C.
- 16 Under a dissection microscope, use stitch scissors to remove the sutures.
- 17 Use tissue-separator scissors to separate the glands from the skin. With the aid of retractors, pull open the skin to better expose the glands. *Healthy glands have lobules and appear pinkish gray. It is rare to detect any damage in the glands from the first surgery. Even if some inflammation occurs in the glands, it does not affect imaging in the submandibular neurons.*
- 18 Using fine forceps to cut connective tissue, locate the SMG anterior to one of the salivary glands.

The SMGs are located along the salivary duct and surrounded by connective tissue. They appear as small translucent beads. Healthy ganglia should have detectable capillaries with blood flow.
- 19 Lift the ganglion onto a small metal platform that is controlled by a micromanipulator.

Apply some tension so that the ganglion stays in place.
- 20 Move the stage from the dissection microscope to an upright imaging microscope. Perfuse the area with warm mammalian Ringer's solution.
- 21 Image calcium transients with a one-photon wavelength of 480 nm (or two-photon excitation at 920 nm) and with a 500- to 550-nm bandpass emission filter.

Labeled cells will have dim background fluorescence that fills the soma compared with nonlabeled cells. Transients are bright and can be seen in the raw movies. See Troubleshooting.

TROUBLESHOOTING

Problem: Fluorescence intensity of infected cells is low.

[Step 21]

Solution: The fluorescence quantum efficiency of G-CaMP2 is only in the range of wild-type green fluorescent protein (GFP) at typical intracellular Ca^{2+} concentrations. As a result, infected cells might be hard to find because they are only slightly brighter than autofluorescent cells. Because excessive excitation leads to bleaching, take averaged images without increasing excitation intensity. Search for cells that are brighter than the average cell. Typical calcium signals in vivo increase the intensity by 40% and last longer than a second, making them easily detectable. Note that bleaching accelerates when calcium (and therefore fluorescence) is elevated.

DISCUSSION

PRV369 provides the capability to reliably detect neuronal activity by expression of a fluorescent calcium indicator protein in intact circuits in living animals. It is isogenic with PRV Bartha strains encoding GFP (PRV152; Smith et al. 2000) and red fluorescent protein (PRV614; Banfield et al. 2003), two of the most frequently used PRV-based viral circuit tracers. PRV369 should therefore be useful in circuits previously elucidated by these tracers. The endogenous activity of PRV369-labeled neurons can be monitored early on with minimal effects caused by infection. However, at later time points in infection, the number of infected cells and calcium transient frequency increases significantly, perhaps indicating cell and tissue responses to infection (Granstedt et al. 2009).

The described approach for labeling and monitoring activity in the SMGs can be generalized to other circuits. Although not all types of circuits have been explored with PRV, to our knowledge PRV has labeled every circuit tested. However, for each circuit the precise time window for reliable imaging must be determined empirically by measuring the time course of infection spread through the circuit. It is best to image cells that are infected early rather than late. Many factors influence the time when optimal imaging can be done. Critical variables include the injection site, infection dose, viral strain, and animal species (Card 1998). When performing intraparenchymal injections of PRV, the injection pressure is an additional important factor. The PRV virion has a diameter of ~200 nm and the width of undisturbed extracellular space in the brain is in the 40–60 nm range (Thorne and Nicholson 2006). When injected at a low rate (1 nL/min), PRV virions remain highly localized at the site of injection. With increasing pressure, the extracellular space widens, increasing the volume, which in turn leads to more dispersed inoculum and more widespread infection.

The time when cell bodies become infected after injection can be estimated using some basic parameters: the number of serial synapses that must be crossed from the point of inoculation to the desired area for imaging, the time to replicate and package PRV, and the distance over which virus must be transported. In cultured neurons, PRV replication and packaging reaches a maximum by ~10 h and virions move in the retrograde direction at ~1 $\mu\text{m}/\text{sec}$, or 3600 $\mu\text{m}/\text{h}$ (Smith et al. 2001). With these assumptions, an approximate formula for how long to wait after inoculation is:

$$\text{Wait time in hours} = [10 \times (\text{number of synapses to cross})] + [(\text{total transport distance over dendrites and axons in } \mu\text{m}) / 3600].$$

Because SMG neurons are difficult to impale for intracellular recordings, PRV369 is valuable for allowing characterization of neuronal activity in a circuit that is otherwise difficult to access by conventional recording methods. Another advantage is the relatively fast calcium-dependent kinetics of G-CaMP2 compared to other indicator proteins, allowing better separation of successive signals in time. In the future, the advent of improved versions of G-CaMP (i.e., G-CaMP3, G-CaMP4) should lead to improvements in signal-to-noise ratio. Alternative calcium-sensor PRV strains such as As1-PRV08 (Boldogkoi et al. 2009)

can also be used with the described protocol after determination of virus-specific characteristics (e.g., time point of experiment after inoculation) and of indicator-specific parameters (e.g., excitation wavelength, filters). In summary, this protocol allows for reliable and sensitive detection of endogenous circuit activity at single cell resolution and early in infection after inoculation with the circuit-tracing PRV369.

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Figure 1.

Example of an SMG infected by PRV369. Spontaneous calcium transients were imaged with two-photon microscopy 48 hours post-inoculation (hpi). Scale bar = 20 μ m. F/F: relative fluorescence change.