Protocol

In Vivo Voltage-Sensitive Dye Imaging of Mammalian Cortex Using "Blue" Dyes

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Optical recording of membrane potential allows simultaneous measurements to be taken from many different locations in the nervous system. This is important in studies of the nervous system in which simultaneous activity can occur at the regional, cellular, and subcellular levels. New "blue" dyes, developed by Amiram Grinvald's group, are a great advance for in vivo voltage-sensitive dye imaging of mammalian cortex. The blue dyes are excited by red light (630 nm) that does not overlap with light absorption of hemoglobin (510–590 nm). This virtually eliminates the heart pulsation artifact.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPE: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Atropine sulfate (40 µg/kg)
Dexamethasone sulfate (1 mg/kg)
Dye RH 1691 (Optical Imaging) (Shoham et al. 1999)
Holding artificial cerebrospinal fluid (ACSF) <R>
Isoflurane (2%–3%; in air)
Sprague–Dawley and Long Evans rats (250–400 g)

Equipment

Calibrating multigas monitor (BCI 9100)
Catheter (16-gauge; over-the-needle)
Imaging setup

The cortex is imaged by a 5× macroscope with a field of view of ~4 mm in diameter. A halogen tungsten filament lamp (12 V, 100 W, Zeiss) is used for illumination. Light is filtered with a 630 ± 15-nm interference filter (Chroma Technology) and then reflected down onto the cortex via a 655-nm dichroic mirror (Chroma Technology). Köhler illumination is achieved through the macroscope. The fluorescence of the dye (~700 nm, Optical Imaging) from the stained cortex is collected via the macroscope, filtered through a 695-nm long-pass filter (RG-695, Edmund Scientific), and projected onto the fiber-optic aperture of the photodiode array. Each detector of the array receives light from a cortical area of ~160 µm in diameter.

Adapted from *Imaging in Neuroscience: A Laboratory Manual* (ed. Helmchen and Konnerth). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Perfusion pump, custom-made (see London et al. 1989)
Silicone oil (high-viscosity; 60,000 cSt) (Sigma-Aldrich DMPS-60M)
Silicone valve grease
Small animal respirator (Harvard Apparatus)
Stereotaxic frame with a regulated heating pad
Surgical adhesive (Vetbond from 3M)
Surgical drill

METHOD

Surgery

1. Pretreat Sprague–Dawley and Long Evans rats (250–400 g) with atropine sulfate (40 µg/kg intraperitoneally [i.p.]) ~30 min before anesthetic induction to reduce mucus secretion.
2. Anesthetize the animals with 2%–3% isoflurane in air.
3. Perform a tracheostomy by inserting a 16-gauge over-the-needle catheter into the trachea.
4. Ventilate the animal using a small animal respirator with isoflurane in room air. Adjust the respiration rate (60–100/min) and volume (2–4 mL) so that inspiratory pressure is ~5 cm H_2O and the end-tidal carbon dioxide is maintained at ~26–28 mm Hg with a calibrating multigas monitor.
5. Place the animal in a stereotaxic frame with a regulated heating pad. As soon as the animal is secure, reduce the isoflurane to 1.5%–2% for craniotomy.
6. Clean the cranial surface thoroughly of soft tissue, and then apply a thin layer of surgical adhesive to prevent dye leakage during staining.
7. Drill a cranial window (~5 mm in diameter). Immerse the drill tip in ACSF to diffuse heat during drilling. Great care must be taken to avoid irritation of the cortex by heat or pressure during this procedure.
8. Carefully separate the bone from the dura. Leave the dura intact to reduce the heart beat movement artifact during optical recording (London et al. 1989; Lippert et al. 2007).

   Irritated brain can appear reddish owing to increased blood flow, which leads to poor staining. Dexamethasone sulfate (1 mg/kg i.p.) may be used 6–24 h before the experiment to reduce the inflammatory response of the dura.

Staining

9. Dissolve the voltage-sensitive dye RH 1691 at 2 mg/mL in ACSF solution.
10. Wash the surface of the dura with ACSF, and then remove the fluid thoroughly by suction. Allow the dura to dry completely for 3–4 min, with gentle air flow, until the tissue becomes very transparent and "glassy."

    This "glassy" appearance is essential for good staining. Drying the dura before staining increases its permeability to the dye.

11. Perform staining through the intact dura. Construct a temporary staining chamber on the surrounding bone using silicone valve grease.
12. Use ~200 µL of dye solution (~2 mg/mL of RH 1691) to stain an area of the dura 5 mm in diameter. During staining, circulate the dye solution continuously using a custom-made perfusion pump (London et al. 1989).

    The pump has a battery-powered gear motor that gently presses the rubber nipple of a Pasteur pipette once every few seconds. The tip of this pipette is placed in the staining solution and performs a gentle, back-and-forth circulation of a small amount of dye (~100 µL). Such circulation is necessary because CSF slowly exudes from the dura and dilutes the dye concentration locally at the dye/dura interface.

13. Stain for 90 min, and then wash the dura with dye-free ACSF for >15 min.

Physical stabilization of the cortex can be further improved by application of high-viscosity silicone oil on top of the dura after the ACSF wash. This silicone oil is replaced every 30 min to 1 h, because exuded CSF can accumulate underneath and hinder the dampening effect.

Sensitivity and Total Recording Time

14. Image using the imaging setup described above.

DISCUSSION

When the cortex is properly stained, the sensitivity of the voltage-sensitive dye recording can be comparable to that of local field potential (LFP) recordings (Lippert et al. 2007). When the LFP is simultaneously recorded with dye imaging, by comparing these two signals, we found most of the peaks in the LFP showed a corresponding event in the imaging signal. There is a high correlation between optical traces that is not caused by light scattering or optical blurring because there is a small timing difference between locations caused by waves propagating in spatiotemporal patterns (Slovin et al. 2002; Petersen et al. 2003; Lippert et al. 2007; Xu et al. 2007). At the light intensity for a good signal-to-noise ratio for sleeplike waves, the total recording time can be up to 1000 sec, broken into 80–100 trials of 10 sec, which is sufficient for many types of experiment.

RELATED INFORMATION

To visualize spatiotemporal patterns in rodent neocortex in vitro, see **Voltage-Sensitive Dye Imaging of Population Signals in Brain Slices** (Baker et al. 2015).

RECIPE

Holding Artificial Cerebrospinal Fluid (ACSF)

Reagent	Final concentration
NaCl	126 mM
KCl	2.5 mM
$CaCl_2$	2 mM
$MgSO_4$	2 mM
NaH_2PO_4	1.25 mM
$NaHCO_3$	26 mM
Dextrose	10 mM

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