Video Article

Optical Recording of Electrical Activity in Guinea-pig Enteric Networks using Voltage-sensitive Dyes

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URL: https://www.jove.com/video/1631
DOI: doi:10.3791/1631

Keywords: Cellular Biology, Issue 34, naphthylstyryl-pyridinium dye, di-4-ANEPPDHQ, enteric nervous system, submucous plexus, myenteric plexus, potentiometric probes, enteric plexuses, neural networks, gut, optical recording, voltage-sensitive dyes

Date Published: 12/4/2009

Citation: Obaid, A.L., Salzberg, B.M. Optical Recording of Electrical Activity in Guinea-pig Enteric Networks using Voltage-sensitive Dyes. *J. Vis. Exp.* (34), e1631, doi:10.3791/1631 (2009).

Abstract

The enteric nervous system (ENS) is a self-contained network with identified functions, capable of performing complex behaviors in isolation. Its neurons (10 to 25 µm in diameter) are arranged in plexuses that are confined to distinct planes of the gut wall [1]; the myenteric plexus can be found between the longitudinal and circular muscle layers, and the submucous plexus between the circular muscle layer and the mucosa. Since the effector systems for these plexuses (transporting epithelium, endocrine cells, immune elements, blood vessels and smooth muscle) are also contained within the gut wall, semi-intact preparations can be dissected that preserve individual components of different reflex pathways. The behavior of the effector systems is controlled by the submucous and myenteric plexuses acting in concert. Therefore, detailed knowledge of synaptic interactions within and between ganglia, and of communication between the plexuses, is essential for understanding normal gastrointestinal function. The ENS, as an intact nervous system, is a unique experimental model in which one can correlate molecular and cellular events with the electrical behavior of the neuronal network and its physiological outputs. Because of the quasi-two-dimensional organization of its plexuses, the ENS is particularly well suited for the study of neural networks using multiple site optical recording techniques that employ voltage-sensitive dyes [2,7,8,9]. We will illustrate here the use of a relatively new naphthylstyryl-pyridinium dye (di-4-ANEPPDHQ) [3] that offers multiple advantages over its predecessors, including very low phototoxicity, slow rate of internalization, and remarkable chemical stability. When used in conjunction with a camera that permits sub-millisecond time resolution, this dye allows us to monitor the electrical activity of all the neurons in the field of view with a maximal spatial resolution of ~ 2.5 µm at 100X magnification. At lower magnification (10X or 20X), the sacrifice of single-cell resolution is compensated by a gain in perspective, revealing the intricacies of the inter-ganglionic circuitry.

Video Link

The video component of this article can be found at https://www.jove.com/video/1631/

Protocol

Part 1: Tissue preparation

1. The submucous and myenteric plexus preparations are isolated by sequential dissection from the small intestines of 150-200g (2-3 week old) Hartley guinea pigs that have been anesthetized by isoflurane inhalation and decapitated. After euthanasia, the small intestine is excised and its contents removed by flushing the lumen with a warm, oxygenated solution. We use Medium 199 supplemented with 25 mM HEPES, 5 mM $NaHCO_3$ and 2 mM Glutamine, and pH adjusted to 7.4 (referred to, from now on, as M199-DM), which has been pre-warmed at 37 °C. All the remaining steps of the protocol, however, will be carried out at room temperature.
2. An intestinal segment, 8-10 cm in length, is transferred to a Sylgard dish containing M199-DM, opened along the mesenteric border, and mounted mucosa side-up using insect pins. It is extremely important to maintain even tension, so that after pinning the tissue exhibits a rectangular shape with the rows of mucosal villi regularly aligned.
3. Using fine Dumont forceps with straight tips, in an almost horizontal position, it is easy to peel the mucosa by grabbing the villi at one end of the preparation and pulling away along the longitudinal axis of the tissue. When the sample is evenly pinned, ribbons of mucosa can be separated in a few individual strokes, exposing the submucosa that contains the submucous ganglia and the submucosal vasculature.
4. For the fine dissection of the plexuses, we recommend using a dissecting microscope with dark-field illumination. Indeed, the pseudo three-dimensional effect created by this type of illumination helps to distinguish the individual layers, thereby preventing or minimizing instrument-inflicted damage.
5. To make a submucosal preparation, we follow the classical procedure of Hirst and McKirdy [4] that requires: a) making a cut in the submucosal layer following the orientation of the circular muscle fibers; this can be achieved using very fine scissors (such as a Moria MC19 or its equivalent); b) lifting the submucosa very gently with the fine forceps, while pushing the circular muscle layer down using the fine scissors; c) cutting along the longitudinal edges as the separation progresses to liberate the submucosa from the rest of the prep; d) making a second cut following the orientation of the circular muscle fibers at a distance from the first one, to define the final size of the submucosal preparation.

The resulting submucosal segment, which is only 30 to 50 µm thick and has a gossamer appearance, can be transferred to another Sylgard dish containing fresh M199-DM, pinned gently and without tension, and kept in an oxygenated chamber for later use.

6. The original gut-segment, now deprived of mucosa and submucosa, and with the circular muscle exposed, can still be used to yield a myenteric plexus preparation. To expose the myenteric plexus, one must eliminate as much as possible of the circular muscle layer. This can be achieved by using the same straight-tip fine forceps employed in previous steps. Bundles of fibers can be pulled gently away, first from one, and then from the other side of the preparation. The exposed myenteric plexus cannot be separated from the longitudinal layer of muscle and the underlying serosa. Therefore, what is called the myenteric plexus preparation is the segment that can be cut along the pins. Because it contains live muscle, it is advisable to pin it very loosely in the dish containing oxygenated M199-DM until the physiological experiment begins.
7. Since the mucosa is the main ENS sensor, and all the effectors are also embedded in the gut wall, it is possible to dissect semi-intact preparations that preserve entire reflex pathways. These custom-designed preparations require combinations of the steps described in 1.3. to 1.6., applied to adjacent areas of the isolated intestinal segment.
8. A few minutes prior to the actual optical experiment, the preparation of choice is mounted in the experimental chamber, making sure that the tension is evenly distributed and the tissue lies completely flat against the Sylgard bottom. This is achieved by holding the specimen with multiple very fine pins that can be positioned all around the perimeter without inflicting tissue damage.

Part 2: Preparation of tissue samples for optical recording

1. (Optional) To prevent background fluorescence from dye binding to residual smooth muscle and connective tissue, both isolated plexuses may be incubated for 30-60 min in M199-DM containing collagenase VII (≤50 U/ml) and Protease IX (≤0.5 µg/ml). Following the enzyme treatment the preparations should be washed with, and maintained overnight in, M199-DM plus 10% animal serum (e.g., equine or bovine) and antibiotics (penicillin, 100U/ml; streptomycin, 100 µg/ml), in a chamber saturated with 95% O_2 and 5% CO_2. This procedure, which we have used for many years because it noticeably enhances the signal-to-noise ratio of the optical recordings, may not be helpful for beginners and should be adopted with caution. Indeed, a preparation damaged during dissection runs the risk of disintegrating during the enzyme treatment. However, even in the absence of the enzyme treatment, we recommend that the preparations be maintained overnight in M199-DM with serum and antibiotics. This step promotes tissue healing after acute dissection damage, and increases the transparency of the sample.
2. Optical recordings of electrical activity cannot be achieved in a preparation in which the muscle is contracting. Therefore, to prevent muscle contraction when recording from the myenteric plexus, a few minutes prior to the optical experiment we add 2 µM nifedipine to the M199-DM bathing the tissue.

Part 3: Staining with di-4-ANEPPDHQ

1. Di-4-ANEPPDHQ is commercially available in lyophilized form. We recommend dissolving it in pure ethanol, making a concentrated stock solution [e.g., 15-20 mg/ml]. This stock, when kept at -20°C, is extremely stable. Indeed, in our laboratory, we just finished the last drop of a stock solution of this dye that we had made almost 6 years ago.
2. The staining solution should be prepared in M199-DM, and should contain 2 µM nifedipine if the protocol requires it. The final concentration of dye can be as low as 5 µg/ml and should not be higher than 50 µg/ml. However, depending upon the concentration of the stock, a certain amount of ethanol will be present in the staining solution and should be taken into account. For example, a staining solution with 50 µg/ml of dye will contain ~0.3% ethanol if made from a 15 mg/ml stock, but close to 1% ethanol if the stock had a concentration of only 5 mg/ml.
3. For staining, the preparation is submerged for 10-30 minutes in the dye solution. After that period, the dye is washed away and replaced by fresh, oxygenated M199-DM. Since di-4-ANEPPDHQ is a membrane-impermeant dye that fluoresces only when in a lipid environment, healthy neurons from a stained enteric preparation should have the appearance of empty balloons.

Part 4: Electrical stimulation

1. Stimulation can be driven by voltage or by current. We use current stimulation, delivered by a computer controlled, 4-channel stimulus generator with a maximum output of 16 mA per channel.
2. The electrode model to be used in a given experiment is determined by the experimental objective. Intracellular pipettes or patch electrodes can be used when the goal is the stimulation of a particular neuron. When the stimulation is intended to activate more than one neuron in a given ganglion, an extracellular electrode touching a connective may be used. To study interconnecting ganglia, large, low-resistance electrodes and/or multi-element electrode arrays can be employed; however, when using current stimulation as we generally do, multi-element arrays become the only option. Many models of extracellular electrodes, as well as some linear electrode arrays, are readily available from commercial sources.
3. Electrodes can be positioned using different types of micromanipulators. To avoid the vibration induced by long and fine barrels, we favor using small micromanipulators on magnetic bases that can be positioned adjacent to the experimental chamber, and supporting the flexible barrels of the tungsten electrodes with thin but very rigid metal bars.

Part 5: Perfusion

We use a 2-channel peristaltic pump with one channel for input of oxygenated solutions, and the other for output. To eliminate mechanical disturbances induced by the perfusion, the pump is turned-off during the acquisition of optical data.

Part 6: Apparatus for multiple site optical recording of membrane potential

1. Optical components:

The apparatus for multiple site optical recording of membrane potential comprises a NeuroCCD-SM camera (RedShirtImaging) and a 1:10 demagnifier / relay lens mounted on the trinocular tube of an upright microscope. The microscope moves on an X-Y translator, independently of a stage that is rigidly fixed to the top of an active vibration isolation table. To minimize airborne vibration, the entire apparatus is surrounded by heavy acoustic curtains and a mylar roof. Epi-illumination is provided by a 100W tungsten-halogen lamp powered by an ultra-low-ripple feedback stabilized power supply. The incident light is made quasi-monochromatic using a heat filter, a high-Q interference filter (HQ530/50) and a 565 nm (50% transmission) dichroic mirror. Fluorescence emission is separated using the dichroic mirror and a long-pass filter (HQ572LP). The intensity of the incident light is adjusted using neutral density filters. Trans-illumination, for bright-field viewing of the preparation is provided by a second 100 W tungsten-halogen lamp driven by a 0-55 V, 1-10 A power supply that is not required to possess any special attributes.

2. High-speed camera specifications:
   The electronic design and the performance characteristics of the NeuroCCD-SM camera have been described in detail [3]. Briefly, it is a cooled, low resolution, precision high-speed camera that uses the back-thinned, back-illuminated Marconi CCD39-01 chip (80x80 pixels). Digitization is 14-bit, and the full frame rate is 2 kHz. However, for specific purposes, one may increase the frame-rate up to a maximum of 10 kHz by using various binning combinations or reducing the number of functional pixels. The camera has the important advantage of extremely low read noise (23 electrons at 2 kHz; 9 electrons at 1 kHz). In addition, its pixels have a relatively large well depth (215,000 electrons), permitting moderate light intensities at high frame rates.
3. Data Acquisition and Analysis Software:
   The optical experiment is controlled by Neuroplex, a software package from RedShirtImaging, that operates as an application within the IDL platform (Interactive Data Language) and includes multiple features necessary for acquisition, display and analysis of the optical data.
4. Additional camera:
   As an integral part of the apparatus, a beam splitter on the trinocular head of the microscope relays an image of the preparation onto a second, high-resolution CCD camera connected to a frame-grabber. The image, which is acquired and stored using Global Lab Image/2 software, can be superimposed onto the display of the sub-millisecond time-resolved optical signals captured by the NeuroCCD-SM camera to generate an accurate map of the spatial origin of the optically recorded voltage signals. In our lab, the frame grabber for this camera resides in a different computer than the one responsible for functional data acquisition. Therefore, the image file has to be transferred from one computer to another, and small adjustments in size and position of the high-resolution image are required before perfect registration of the two cameras is achieved.

Part 7: Optical experiments

1. Prior to each functional recording, we acquire the high-resolution black and white image of the region from which we plan to record electrical activity by optical means (as indicated in 6.4). This image, when superimposed onto the pixel-map of the high-speed camera, helps us to identify the structures that are the source of the optical signals.
2. In the appropriate system configuration, Neuroplex (see 6.3) also controls the shutter, and triggers the electrical stimulation. Since the high-speed data acquisition requires considerable computer power, it is advisable, if at all possible, to use a dedicated computer with a large amount of memory and a high capacity hard drive. That, however, is not trivial. Our own system, e.g., already several years old, employs data acquisition boards that are too large to fit in state-of-the-art computers. As a compromise, we adopted and dedicated to our optical system a DELL Precision 390 equipped with a 500 GB hard drive and 4 GB of RAM. This computer allowed us to keep our older, but adequate, A/D and D/A boards, while still providing sufficient power to run our experiments.
3. As soon as the acquisition is over, Neuroplex can display the results in multiple complementary formats, some of which are detailed below:
   a. An area of the screen called page display shows the crude image of the preparation as captured by the 80X80 high-speed camera as an individual frame. Superposition onto this pixel-map of the high-resolution image acquired with the second camera (as explained in 6.4 and 7.1) refines the operator s ability to identify areas of interest.
   b. When pixels of this page display are selected under mouse control, Neuroplex automatically converts their optical outputs into traces that show the magnitude of the optical change registered by those pixels as a function of time. (e.g., ΔF/F vs. time). The operator can decide whether these traces should reflect the output of individual pixels, or, rather, the space-averaged output of multiple loci over a given region of the preparation.
   c. The data can also be displayed in movie-format.
      To ensure that all the information contained in individual experimental runs is fully extracted, however, we favor off-line analysis of optical data. Representative results of this analysis are available.

Part 8: How to achieve technical quality in an optical experiment

The technical success of an optical experiment is measured, ultimately, by the signal-to noise ratio of the recordings. Furthermore, a good signal-to-noise ratio stems not from an epiphany but from a journey. As in an opera performance, the final outcome is determined by a multiplicity of factors that interact synergistically. Thus, to achieve a technically sound optical experiment, it is essential to refine, independently, the optical apparatus and the skills required for each individual step of the protocol. What follows is a list of potential trouble-areas, and some suggestions for avoiding problems not always anticipated [5]:

1. Noise sources
   Mechanical and optical stability are crucial when recording electrical activity with voltage-sensitive dyes, because the fractional change (ΔF/F) reflecting physiological responses is relatively small (< 10% / 100 mV in most of the gut recordings) and a noisy trace always augurs a bad outcome. Therefore, to optimize the signal-to-noise-ratio, it is imperative to eliminate, or, at least, to minimize, the contributions of the two worst offenders: a) mechanical vibrations, and b) light source fluctuation.
   a. Mechanical vibrations are generated by a variety of sources as diverse as setup configuration, building stability, proximity of motorized equipment, air-currents, acoustic noise, fluid motion, or particles floating in the bathing solution. A good vibration-isolation system, and acoustic curtains for extreme cases (see, e.g., description in 5.1), make a big difference and are well worth their cost. In addition,

common-sense approaches, such as filtration of solutions used in the experiment, or the use of magnetic bases for small, moveable parts of the setup, are quite effective.

b. Light sources are most stable at quasi-maximal intensity. Therefore, one should set a current-limit for the power supply, and, when necessary, reduce the light intensity by inserting neutral-density filters in the light path instead of by reducing the voltage. Arc lamps are intrinsically noisy. Tungsten-halogen bulbs or light-emitting diodes are much more stable and should be the preferred choice [6].

2. Animal age
Dissections should be done using the gut of young animals because, as the connective tissue within the gut toughens with age, it becomes more difficult to separate individual tissue layers without inflicting major damage on the neurons of the two plexuses, whose afferent and efferent processes may cross layer boundaries and/or project for quite a distance along the longitudinal axis of the gut. This advisory, which can be disregarded when studying intra-ganglionic connections, is of paramount importance when the experimental objective is the analysis of inter-ganglionic circuits or the activation of reflex pathways.
3. Dye internalization
It is important to use as low a dye concentration as possible, and to minimize unnecessary light exposure. Although di-4-ANEPPDHQ has proven to be much less phototoxic than other dyes, it will get internalized, eventually, as the health of the preparation deteriorates. Dye internalization diminishes the signal-to-noise ratio by increasing dramatically the background fluorescence [3].
4. Electrode maintenance
    1. Electrodes should be washed carefully and thoroughly after each experiment. Organic deposits at the tips of tungsten electrodes increase electrode resistance and prevent stimulation.
    2. The insulation of commercially available tungsten electrode is very delicate and prone to damage. A leak in the insulation will shunt current, compromise the effectiveness of the stimulation and jeopardize the success of the experiment.

5. Perfusion perils
Never leave the set-up unattended while the perfusion is on. If the in-flow and out-flow of the peristaltic pump are not properly balanced, two catastrophic events may take place:
    a. A flood may cause irreversible damage to the microscope.
    b. The preparation may dry out during the experiment.

6. Software hassles
It is essential to familiarize oneself with the software that controls acquisition as well as analysis. Fast data-acquisition and/or the creation of very large files tend to drive the system to the brink of breakdown, and Neuroplex, which is quite unforgiving, will crash relentlessly unless its boundary conditions are respected. Subtleties within the records, easily missed when the analysis is confined to limited regions of interest, often reveal themselves in movie displays of the data.

Discussion

This protocol has been written with two goals in mind. The first one is to persuade other investigators that, thanks to the many technological advances of the last decade, optical recording of electrical activity using voltage-sensitive dyes has become one of the most powerful, reliable and affordable methodologies for studying intact neuronal networks; indeed, it could be easily implemented even in a laboratory having modest resources. The second goal is to promote the enteric nervous system as a unique experimental model in which to correlate molecular and cellular events with the electrical behavior of the two neuronal plexuses and their biological outputs.

Acknowledgements

We would like to thank D.A. Coulter (CHOP) for allowing us to use his 2-photon microscope to acquire the 2-photon images shown at the beginning of the video, and H. Takano for sharing with us his technical expertise.

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