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A stereo-compound hybrid microscope for combined intracellular and optical recording of invertebrate neural network activity

William N. Frost, Jean Wang, and Christopher J. Brandon

Dept. of Cell Biology and Anatomy The Chicago Medical School Rosalind Franklin University of Medicine and Science

Abstract

Optical recording studies of invertebrate neural networks with voltage-sensitive dyes seldom employ conventional intracellular electrodes. This may in part be due to the traditional reliance on compound microscopes for such work. While such microscopes have high light-gathering power, they do not provide depth of field, making working with sharp electrodes difficult. Here we describe a hybrid microscope design, with switchable compound and stereo objectives, that eases the use of conventional intracellular electrodes in optical recording experiments. We use it, in combination with a voltage-sensitive dye and photodiode array, to identify neurons participating in the swim motor program of the marine mollusk *Tritonia*. This microscope design should be applicable to optical recording studies in many preparations.

Keywords

optical recording; voltage-sensitive dyes; *Tritonia*; photodiode array; microscopy; electrophysiology; neural networks

Introduction

Many of the most interesting issues regarding brain function concern how networks of neurons process information, store memory, and generate behavior. One powerful technique for observing neural network activity is optical recording with voltage-sensitive dyes. These lipophilic molecules bind to neuronal membranes and change their light absorbance or fluorescence in response to changes in the cell's membrane potential (Grinvald et al., 1988). By positioning a camera or photodiode array at the image plane of the microscope, one can monitor the activity of large numbers of neurons simultaneously in real time, providing a view of network activity impossible to achieve with sharp electrodes alone.

Invertebrate nervous systems are highly attractive for optical recording. Their neurons are both large, and are arranged on the surface of ganglia, making it possible to simultaneously image the activity of large numbers of individually-identifiable neurons. Invertebrate preparations whose neural networks have been studied with optical recording include *Aplysia* (Nakashima et al., 1992; Zecevic et al., 1989; Zochowski et al., 2000), *Tritonia* (Brown et al., 2001), *Helix*

Corresponding author: William N. Frost, Dept. of Cell Biology and Anatomy The Chicago Medical School, Rosalind Franklin University of Medicine and Science 3333 Green Bay Road North Chicago, IL 60064, Tel: (847) 578-8634 and Fax: (847) 578-3253, Email address: william.frost@rosalindfranklin.edu

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(Nikitin and Balaban, 2000), *Lymnaea* (Kojima et al., 2001), *Navanax* (London et al., 1987), *Limax* (Gelperin and Tank, 1990), lobster (Wachowiak and Cohen, 1999), leech (Briggman et al., 2005) and silkworm (Ai and Kanzaki, 2004). Neurons in some of these invertebrates, such as *Tritonia diomedea*, have somata that are fully invaded by active, full-size action potentials, a special advantage for voltage-sensitive dye recording.

While the use of optical recording in invertebrates is increasing, few such studies have incorporated conventional intracellular electrodes, which we believe may be limiting the potential usefulness of the technique. Without intracellular recording, one may optically record activity in large numbers of neurons, but the identities of these neurons, or even their general category (eg., sensory neuron vs. interneuron vs. motor neuron) typically remains unknown.

A key hindrance to using sharp electrodes in optical recording studies is that different optical systems are best-suited for the two techniques. Conventional neurophysiological studies in invertebrate networks typically involve the visually-guided placement of multiple sharp electrodes into specific different neurons. This is a demanding task, most easily accomplished with stereomicroscopes because of their upright images, stereo view, and comfortable working distances. On the other hand, optical recording typically requires compound microscopes, with illumination systems and objectives better suited to delivering maximum light to the imaging device. Unfortunately, the short working distances, typically inverted images, and particularly the lack of stereopsis of compound microscopes makes working with multiple intracellular electrodes so difficult that few use them routinely in optical recording experiments (see however, (Briggman and Kristan, 2006; Taylor et al., 2003)).

Here we have addressed this problem by constructing a hybrid microscope having the high intensity illumination system of a compound microscope, and a stereomicroscope-based optical system with switchable stereo and compound objectives. We demonstrate the feasibility of this design for combined optical and conventional intracellular recording in the marine mollusk *Tritonia diomedea*.

Materials and Methods

Construction of the hybrid microscope

To construct the hybrid microscope (Fig. 1) we first cut through the body of a Nikon Optiphot compound microscope we had already used to obtain successful optical recordings. This removed the head and lens turret, leaving intact the base diaphragm, secondary collector lens and condenser of the original light path. An aluminum plate with an attached upright mounting post was bolted to the top of the sawn-off stump, onto which was mounted a Zeiss SV-11 stereomicroscope with an attached lens-switching device (Combizoom 400, Kramer Scientific, Valley Cottage, NY). The lens switcher allows the user to view the preparation with either a standard stereomicroscope objective, which provides the stereopsis and generous working distance ideal for intracellular recording, or up to two different compound microscope objectives, for the higher magnifications and light-gathering ability needed for optical recording. In our version of the Combizoom 400, the lenses are shifted along a front-to-back linear axis, minimizing interference with micromanipulators and other devices situated on either side of the preparation. We then added a Burleigh Gibraltar fixed stage platform (EXFO, Ontario, Canada), onto which were mounted a recording chamber, two micromanipulators (Model MX-130, Siskiyou, Grants Pass, OR) with attached single-axis hydraulic heads, a cooling device for the saline perfusion, and a fiber-optic light for epi-illumination used at times during intracellular penetrations. The microscope was mounted on the Gibraltar platform's motorized translator, allowing the user to move the microscope independently of the electrodes and preparation. This made it possible to penetrate identified neurons in one location, and then shift the view to allow optical recording from various other regions (eg, Fig. 4).

Optical Recording

In initial pilot studies we tested the FRET-based voltage-sensitive dye method developed by Kristan and colleagues to record network activity in the leech (Briggman et al., 2005; Briggman and Kristan, 2006; Cacciatore et al., 1999). Unfortunately, the FRET dye pair used in their studies preferentially stained non-neuronal tissue in *Tritonia*, making it unsuitable for our purposes. We then switched to the voltage-sensitive absorption dye RH-155 (Anaspec, San Jose, CA), which had already been shown to work in *Tritonia* (Boyle et al., 1983; Brown et al., 2001). RH-155 has the additional advantage that its fast response time readily resolves individual action potentials. In our work we use a RedShirtImaging PDA-III photodiode array (RedShirtImaging, Decatur, GA) because its spatial and temporal resolutions (464 photodiodes plus 8 channels of conventional electrophysiology sampled at 1.6 kHz) were well-suited to resolving action potentials in individual neurons in *Tritonia* ganglia. Data acquisition and analysis were performed with the system's Neuroplex software, which allows a precisely aligned image of the preparation to be superimposed on the optical data (Figs. 2 – 4), simplifying the task of locating the neurons that give rise to the optical signals. Optical data were first AC-coupled and amplified in the PDA-III hardware, and were then displayed together with the sharp electrode electrophysiology traces in Neuroplex, where they were further processed with software filters to yield the traces shown in the figures. All traces are of un-averaged data from single acquisitions.

The light reaching the photodiode array originated from an 12V 100W tungsten-halogen lamphouse (Nobska lamphouse, Opti Quip, Highland Mills, NY) powered either by an Opti Quip Model 2100 12V DC power supply or a Power Master 16V racing car battery fitted with a Power Master Step-Down Module that yielded 14.5 volts (Batterycentralmall.com). The battery source had the benefit of providing brighter light to the array, with no 60Hz component. During imaging, the light emitted from the lamphouse passed through a 725/50 band pass filter (Chroma Technology, Rockingham, VT) before entering the back of the Nikon base, where it then passed through the microscope's adjustable base diaphragm and secondary collector lens, a computer-controlled electronic shutter (Model VS35, Vincent Associates, Rochester, NY), a Nikon 0.9 NA substage condenser, the *Tritonia* ganglion preparation, a 20X, 0.6 NA objective lens (Kramer Scientific), and the right hand optical path of the SV-11 stereomicroscope body (Fig. 1B). Finally, a Zeiss phototube passed 100% of the light either to the photodiode array or to the right-hand eyepiece, where the preparation was viewed and focused with a USB microscope camera (Moticam 1000, Motic Instruments, Richmond, B.C., Canada) set parfocal to the photodiode array.

RH-155 was dissolved at 5.4 mg/ml in 100% EtOH, and then aliquoted into 0.3 mg quantities, air-dried in the dark, and stored at -25°C. In our initial experiments the dye was perfused over the brain at 11°C at a concentration of 0.2 mg/ml in saline for 15 min, and then at 0.02 mg/ml at 11°C for the duration of the experiment. In later experiments the initial staining time was lowered to 5 min, which yielded brains with higher excitability (more brains exhibited swim motor programs upon PdN3 stimulation, and the motor programs had higher numbers of cycles). We were able to elicit and optically record several 35s motor program records over 2 – 3 hours, indicating that, under the conditions used, RH-155 has little or no toxicity in our preparation (see also (Parsons et al., 1991)). Saline was either Instant Ocean (Aquarium Systems, Mentor, OH) or filtered natural sea water. To minimize vibration at the air/water interface, we imaged either through a coverslip resting on the solution surface, positioned to leave room for intracellular electrodes, or through a custom-made water immersion cap fitted to our 13mm working-distance 20x objective lens.

Conventional intracellular recording

Intracellular recordings were obtained with glass electrodes filled with 3M KCl. Signals were amplified with intracellular amplifiers (Model IX2-700, Dagan Corporation, Minneapolis, MN) and then digitized by the RedShirtImaging acquisition system, which displayed them with the optical data on a Windows-based computer. Swim motor programs were elicited by stimulating peripheral nerve PdN3 with 2 ms pulses at 10 Hz for 1-2 s using a polyethylene suction electrode. The room was darkened to minimize photobleaching while the dye was in use. During penetration of neurons with intracellular electrodes, the stained preparation was trans-illuminated through a green filter to avoid photobleaching of the dye. Brief periods of white light epi-illumination were used at times to facilitate recognition of landmarks and identified neurons already known to us from our conventional sharp electrode studies.

Tritonia brain preparation

Animals (5 – 50g) were obtained from Living Elements (Vancouver BC, Canada) and maintained either at 11°C in a recirculating artificial seawater system at our home institution, or in 12° C natural sea water at the Marine Biological Laboratory, Woods Hole, MA. Brains were dissected from the animals and pinned to a thin layer of Sylgard (Dow Corning, Midland, MI) covering the coverslip bottom of the recording chamber. After removal of the connective tissue sheath overlying the ganglia, the brain was kept at 11°C using saline passed through a feedback-controlled in-line Peltier cooling system (Model SC-20, Warner Instruments, Hamden, CT) using a peristaltic pump (Model 720, Instech Laboratories, Plymouth Meeting, PA). Saline temperature in the recording chamber was monitored with a Bat-12 thermometer fitted with an IT-18 microprobe (Physitemp Instruments, Clifton, NJ).

Results

We were not sure at the outset whether a stereomicroscope-based imaging setup would have the sensitivity needed for optical recording. With signal-to-noise proportional to the square root of the light intensity reaching the detector, and with the light first passing through a thick, pigmented ganglion, one challenge was to get the maximum amount of light to the array, short of saturating the photodiodes. This was achieved by careful positioning of the bulb, lamphouse mirror and condenser, by over-volting the tungsten-halogen 12V bulb with a 14.5V battery source, and by using brains from small (5 – 50 gm) animals, which have thinner, less-pigmented ganglia. A second challenge involved minimizing vibration-related noise, which could be substantial, even with the setup mounted on an air table. This was achieved via the Gibraltar fixed stage, the use of custom-machined support posts that helped to stabilize the stereo microscope, and by fitting our 20X lens with a custom-machined water-immersion cap.

In its current configuration, the setup can resolve spike activity in large numbers of neurons that contribute to the *Tritonia* swim motor program. Figure 2 shows simultaneous recordings from 36 photodiodes during a swim motor program elicited by a 1s shock to PdN3, a peripheral nerve that contains axons of afferent neurons that trigger the swim. Based on prior studies, most or all of the imaged rhythmically active neurons in this ganglion are likely to be members of the dorsal and ventral swim flexion neuron groups (DFN, VFN). These neurons receive the output of the swim central pattern generator and, in turn, send axons to the periphery to elicit the alternating dorsal and ventral body contractions of the swim.

The primary purpose of our hybrid microscope design was to facilitate the use of sharp electrodes in optical recording studies of neural networks. A common expected application would be in searches for new neurons. Therefore, in one experiment we imaged the ventral side of the pedal ganglion, the “bottom side” of the brain that has been little-explored in almost 40 years of work on *Tritonia*. With the brain pinned out ventral side up, we recorded the activity

of a large number of neurons, all new to us, that fired in phase with one another during the swim motor program. Switching to the stereo objective, we then chose one of the optically-identified neurons and impaled it with an intracellular electrode. A second swim motor program was then elicited and recorded both optically and intracellularly (Fig. 3A). Comparing traces, the impaled neuron was seen to fire one-for-one with the optical recording obtained from the photodiode positioned directly over it. To further confirm that the optical and intracellular recordings were from the same neuron, we then used intracellular current to elicit a train of action potentials in the impaled neuron, and these were found to appear one-for-one as spikes in the corresponding optical recording (Fig. 3B). This experiment demonstrates the suitability of the hybrid microscope for locating new neurons that can then immediately be penetrated and characterized with intracellular electrodes. While a previous sharp electrode study encountered a few neurons that burst in the swim rhythm on the ventral surface of the pedal ganglion, these were described as a continuation of a group located on the edge of the dorsal surface (Hume et al., 1982). The neurons imaged here, confirmed by our penetration of one with a sharp electrode, are located widely over the surface of the ventral pedal ganglion, and therefore appear to be newly described. In addition to the above example, we have also used this approach to identify two new members of the escape swim network (to be described in a separate publication). Thus we have found the process of impaling neurons identified optically in the same preparation to be a straightforward procedure.

The above experiment also contained an unanticipated observation – two neurons that appeared to fire in response to the driven neuron (Fig. 3B). While some dorsal pedal ganglion swim motor program neurons have been shown to indirectly excite one another in intact animal preparations (Willows et al., 1973), such interactions were not similarly observed in isolated brain preparations (Hume and Getting, 1982). Our present observation indicates that some ventral pedal swim neurons that fire together may excite one another through CNS connections, although additional experiments are needed to confirm this.

In 3 preparations, we used this approach to localize pedal ganglion neurons that were either excited or inhibited by cerebral ganglion cell 2 (C2). This member of the swim central pattern generator sends an axon across the central commissure to monosynaptically excite or inhibit several flexion neurons in the contralateral pedal ganglion (Hume and Getting, 1982). After penetrating C2 under the stereo objective, we switched to our compound objective and moved the microscope to image the contralateral dorsal pedal ganglion. We then acquired optical data while using intracellular current to drive action potentials in C2 (Fig. 4). The optical recording revealed the XY ganglion locations of several neurons excited, and several others inhibited by the C2 train, demonstrating the potential usefulness of this technique in searches for new neurons in *Tritonia* and other invertebrate networks. This optical result is consistent with C2's known synaptic connections to pedal ganglion flexion neurons (Hume and Getting, 1982; Snow, 1982). In all such experiments we were able to switch lenses without losing impaled neurons, even with the smallest members of the swim circuit, the 40 - 50 μm diameter S-cells. In these initial experiments with the hybrid microscope, we optically recorded neuronal responses to intracellular stimulation of a number of identified neurons, including the S-cells (1 preparation), TGN cells (2 preparations, (Getting, 1976)), C2 (3 preparations, eg, Fig. 4), and ventral pedal ganglion flexion neurons (1 preparation, Fig. 3B).

Discussion

This report describes our construction of a hybrid microscope with switchable stereo and compound objectives, and demonstrates its usefulness for combined intracellular and optical recording of neurons in *Tritonia diomedea*. As hoped, the hybrid microscope design made it much easier to use intracellular electrodes in combination with optical recording than when using a traditional compound microscope. Here we used it to: 1) record the *Tritonia* swim

motor program, showing that our recording setup has sufficient sensitivity, and that our staining procedure had no serious ill-effects on network activity; 2) optically identify neurons of interest, and then switch to a stereo objective to penetrate and identify them with intracellular electrodes; and 3) penetrate neurons of interest with sharp electrodes and then switch lenses to optically identify the ganglion locations of neurons they excite or inhibit. We found that we were able to switch lenses without damaging already-impaied neurons, even with the smallest members of the swim circuit. Taken together, these results establish the usefulness of the hybrid microscope design for combined intracellular and optical recording of invertebrate neural networks.

Being able to easily combine optical and conventional sharp electrode recording has the potential to significantly facilitate the task of circuit mapping – a rate-limiting step in most studies of identified neuronal networks. For example, by using a sharp electrode to drive action potentials in a known neuron, one can readily identify the exact XY locations of neurons whose firing is modified in response (Figs. 3, 4). A second electrode can then be used to locate these neurons, which, via their connections with already known neurons, can be integrated into the existing circuit diagram. While such an approach is simple in principle, to our knowledge just one laboratory has used it to find new neurons in 30 years of work with voltage-sensitive dyes in invertebrates (Briggman and Kristan, 2006; Taylor et al., 2003). A related advantage of combining intracellular and optical recording stems from the fact that many key neurons, such as VSIB, DRI and PI 9 in *Tritonia* (Frost and Katz, 1996; Frost et al., 2003; Getting, 1983), reside in cell layers below the surface. With its ability to see deep into the tissue (Zecevic et al., 1989), optical recording with trans-illumination should be able to guide sharp electrodes to such “hidden” neurons.

In summary, the paucity of published studies combining conventional intracellular and optical recording may in part be due to the difficulty of penetrating multiple specific neurons with compound microscope optics in the highly three-dimensional terrain of invertebrate ganglia. To this end, we have developed a hybrid microscope design that combines desirable features of both compound and stereomicroscope optical systems. This microscope design should facilitate the combined use of conventional and optical recording of neuronal activity in invertebrate nervous systems.

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Figure 1.

Configuration of the hybrid microscope. **A.** The optical system consists of the lower half of a Nikon Optiphot compound microscope, a Kramer Instruments Combizoom 400 lens switching device mounted on a post affixed to the Nikon base, and a Zeiss SV-11 stereomicroscope body. In our configuration, the Kramer device allows the user to switch between a 1.6X stereomicroscope objective, used for penetrating neurons with intracellular electrodes, and a 20X 0.6NA objective, used for the optical recordings. The entire microscope sits atop a motorized microscope translator. The photodiode array is in the box attached to the phototube at the top of the microscope. **B.** The component parts of the microscope, showing the light path to the photodiode array.

Figure 2.

Optically-recorded swim motor program activity. **A.** In all experiments a camera image of the preparation was aligned with the photodiode array so that we could determine the precise XY position of the neurons imaged by each photodiode. Each short line segment in the hexagonal grid indicates the position of a single photodiode. **B.** Simultaneously recorded optical data from the 36 photodiodes marked red in Panel A. Many different neurons are evident in this small sampling from the 464 total traces acquired, including several that fired in rhythmic bursts during the swim motor program. The vertical lines in the traces correspond to individual action potentials. The 36 traces begin with the top left red photodiode in Panel A, and then proceed left-to-right down the display of red photodiodes, with the final, bottom trace corresponding to the lower-right red photodiode. Neurons large enough to be detected by more than one

photodiode appear in multiple traces. The vertical scale bar in all figures represents the fractional change in light intensity recorded by the photodiodes.

Figure 3.

Use of optical recording to locate new neurons that were then impaled with intracellular electrodes. **A.** In this experiment, the 20X compound lens was first used to optically record from the ventral side of the pedal ganglion during a swim motor program (not shown). Several photodiodes detected neurons that fired in the rhythmic bursts characteristic of the motor program. We then switched to the stereo lens, impaled the surface neuron located beneath one of the active photodiodes, and then switched back to the 20X lens and recorded the swim motor program shown here. The impaled neuron (intracellular trace) fired one-for-one with the action potentials recorded by the photodiode positioned over it (bottom optical trace). **B.** The impaled neuron was then driven with intracellular current injection and the same photodiode detected its action potentials (bottom optical trace), confirming that we had indeed penetrated the neuron identified optically. In addition, two of the optically-recorded neurons that fired with this neuron in the swim motor program shown in Panel A appeared to be excited by it (1st and 6th optical traces).

Figure 4.

Use of optical recording to locate neurons excited and inhibited by an impaled neuron. Here we began by impaling swim central pattern generator neuron C2 using the stereo objective lens. We then switched to the compound objective and moved the microscope to view the contralateral pedal ganglion. An optical recording acquired while driving C2 with intracellular current (10 Hz, 2 s) revealed the XY locations of several pedal ganglion neurons (arrows) that it either excited or inhibited.