

A method of combined single-cell electrophysiology and electroporation

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Abstract

This paper describes a method of extracellular recording and subsequent electroporation with the same electrode in single retinal ganglion cells in vitro. We demonstrate anatomical identification of neurons whose receptive fields were measured quantitatively. We discuss how this simple method should also be applicable for the delivery of a variety of intracellular agents, including gene delivery, to physiologically characterized neurons, both in vitro and in vivo.

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1. Introduction

Electroporation is a method for injecting various molecules into cells, including genetic markers and histological dyes. The basic element of electroporation is the application of voltage pulses across cells in culture or in situ, which serve to first form small pores in the cell membrane and then to transfer a charged entity into the cell interior. The magnitude and duration of the voltage pulses are chosen according to the specific protocol, including cell type, tissue geometry and injected molecules, mainly so that they are large enough to transiently induce pores of sufficient size to allow passage of the charged molecule or molecules, but not so large that the membrane, and thus the cell, is destroyed.

Electroporation of individual identified cells is a more recent technique, and has been applied in in vitro (Rae and Levis, 2002; Rathenberg et al., 2003) and in vivo (Haas et al., 2001; Dezawa et al., 2002) protocols. The technique described here takes advantage of the fact that the whole-cell patch type electrode used in the single-cell electroporation protocol is essentially identical to those used in the “loose-patch” (Neher and Sakmann, 1983) protocol that allow high signal-to-noise ratio recordings of extracellular action potentials from the target neuron. Thus, with a simple modification of the associated electronics, elec-

trication and spike recordings may be combined in the same cell.

2. Materials and methods

Isolated intact retinæ from albino New Zealand rabbits were obtained using standard methods. Briefly, rabbits were dark-adapted overnight prior to surgery. Under dim red light, bilateral enucleation was made immediately after euthanasia by intravenous injection of Ketamine 35 mg/kg and Xylazine 5 mg/kg. After removal, each eye was placed in iced Ames medium (Sigma). One eye was used immediately for the retinal preparation, and the other was kept under refrigeration for use within 2–4 h. To obtain the retinal tissue, an eye was hemisected in cold Ames medium, and the eye cup bisected perpendicular to the visual streak, just skirting the optic disc. One to four retinal pieces were harvested from the eyecup by gently pulling the tissue away from the pigment epithelium. All manipulations of the removed eye were made under visual light control with the aid of a dissecting microscope. Retinal pieces not used immediately for recording were stored for later use (up to 4 h after enucleation) in iced Ames medium, in the same manner as the intact second eye. Retinal pieces (typically 3 mm × 3 mm) were glued to a cover-slip photoreceptor side down with 10 mg/ml poly-L-lysine solution. A ~5 µl aliquot of adhesive was spread across a thoroughly cleaned cover-slip and allow to dry. The prepared cover-slip was then held just below the surface of a Petri dish containing cold Ames medium and a retinal piece. The retinal

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piece was then pulled gently onto the cover-slip, until one edge reached the top edge of the cover-slip. The cover-slip with retina was then gradually drawn out of the Ames medium so that the retina adhered to the glass without wrinkles, and then placed into a recording chamber, cover-slip side down, which was fitted with a glass bottom. The recording chamber was perfused at approximately 1 ml/min with oxygenated (95% O₂, 5% CO₂) Ames medium, maintained between 35 and 37 °C with an in-line heater, and mounted on the stage of a DIC epi-fluorescence microscope (Leica model DMLFSA). Visual stimuli were generated on a standard computer monitor (refresh rate 60 Hz), and presented through the microscope condenser by interposing a prism between the condenser and the microscope illuminator. Stimuli were generated with the VisionEgg software package (www.visionegg.org), and stimulus control and data acquisition were accomplished with in house software written in LabView (National Instruments).

Standard whole-cell patch type electrodes (4–6 MΩ) were mounted in a standard patch electrode holder, and the electrode filament directly connected to the input of an AM Systems differential amplifier (model 1700). This particular amplifier includes both recording and stimulus mode for each channel; the stimulus input was supplied by the output of a Cibertec (model CS 20) stimulation amplifier, controlled by the front panel switches.

Under DIC visualization retinal ganglion cells were exposed by touching the inner limiting membrane (ILM) with the tip of a whole-cell patch electrode filled with Ames medium (“surgery pipette”), maintained with a pressure of 20–40 mmHg to create a steady outflow of solution from the pipette. The tip was advanced until it made a slight indentation in the ILM, and the microscope stage was lightly tapped by hand causing the pipette tip to oscillate and pierce the ILM. Once the ILM was pierced, the pipette solution could be seen to invest the ganglion cell layer and partially isolate neuronal somata.

The surgery pipette was then advanced in order to contact a chosen cell, making a small indentation in the soma. At this point the pressure was removed and a slight vacuum (–20 to –40 mmHg) applied in order to bring the soma membrane into the pipette tip. Light responses were then verified by presenting large flashed spots (100% contrast on a grey background, 1–2 Hz flash rate, 5000–10,000 μm diameter on the retinal surface). If no light response was observed, then the pipette was withdrawn slightly and the procedure repeated on another cell body. Once light responses were verified, the surgery pipette was removed. A second whole-cell patch type pipette, filled with Ames medium and 0.2 mM Alexa Fluor 568 (Molecular Probes), was then installed in the electrode holder, and low (~10 mmHg) or zero positive pressure applied. This pipette was advanced through the slit in the ILM made by the surgery pipette, and positioned at the cell body for which the light responses were obtained. Slight suction was again applied, light responses were verified and more complete receptive field measurements made, including the random presentation of full field 100% contrast sinusoidal or square gratings (2–10 cycles, 1–4 Hz temporal frequency, 500–2000 μm spatial period on the retinal surface) moving in different directions (steps of 45°, 5–10 trials for each direction). Once the receptive field measurements were com-

plete (duration between 5 and 20 min), the amplifier was placed in stimulus mode. A 200 Hz train of 20 constant amplitude voltage pulses (10 V amplitude, 1 ms duration) was then applied by the Cibertek stimulator to make the electroporation. During the electroporation stimulus the pipette and soma were carefully observed to verify the pulsatile movement of the soma membrane in response to the voltage pulses. After electroporation, the infra-red illumination was removed, and the epi-fluorescent illumination presented for 1–5 s in order to verify transfer of the Alexa Fluor 568 to the cell body and to capture images from the video camera. If soma filling was not seen, the electroporation step was repeated under IR illumination, either with the same electroporation parameters, or with an increase of typically 20% of the voltage amplitude of the pulses. In most cases, however, once the optimal electroporation parameters had been found soma filling was accomplished in the first electroporation step.

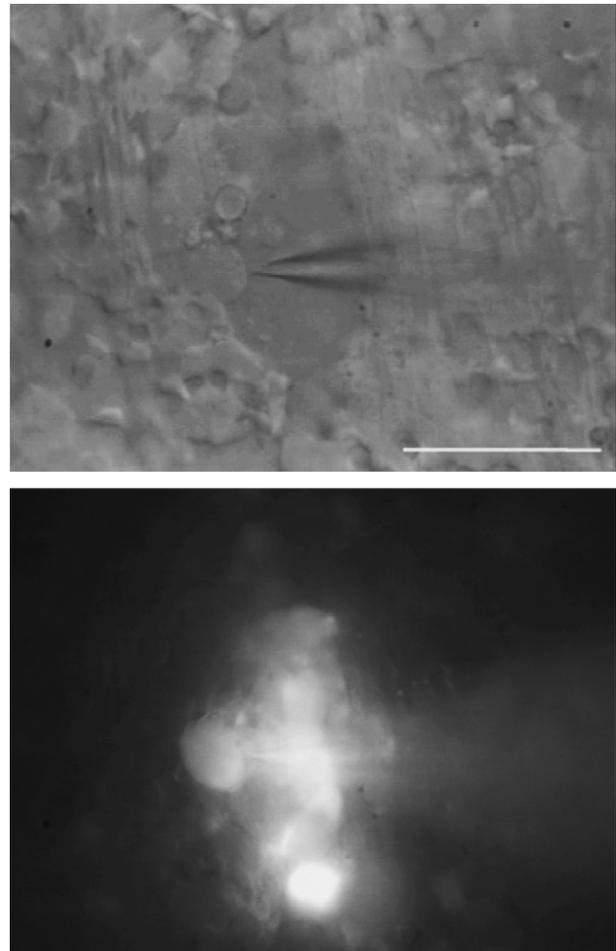


Fig. 1. Retinal ON ganglion cell soma filled with Alexa Fluor 568 under infra-red DIC illumination (top), and epi-fluorescent illumination (bottom). The patch electrode has been retracted after the electroporation so that it is no longer touching the cell body. Note the cleared area to the right of the cell body, made with the “surgery” pipette prior to the placement of the combined electrophysiology and electroporation pipette. Under the epi-fluorescent illumination, Alexa Fluor is also seen in the extracellular space as a result of out-flowing during the initial approach of the electrode. Scale bar 50 μm.

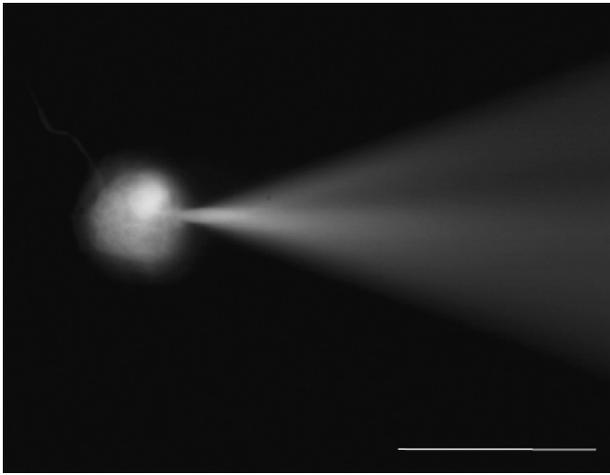


Fig. 2. Retinal ON ganglion cell soma immediately after electroporation with Alexa Fluor 568. The axon is seen leaving the soma at the top left. Scale bar 50 μm .

Electroporation was also attempted with -10 , -5 and 5 V amplitude pulses, without success. After the electrophysiology–electroporation protocols were completed for several cells in a given retinal piece, the tissue and attached cover-slip were removed and fixed in 4% paraformaldehyde solution for 2 h at room temperature. Fixed tissue was then washed with phosphate buffer and mounted with Vectashield mounting medium (Vector) and images made with a Zeiss PASCAL5 confocal microscope (Zeiss, Heidelberg, Germany). Cells were drawn from the confocal image stacks with the aid of standard commercial software (Adobe Photoshop 5.0).

3. Results

We were able to record visual responses and electroporate several ganglion cells from each mounted retinal piece over a period of 1–3 h. Visual responses remained robust over this time, despite exposure to the high intensity epi-fluorescent illumination used to verify each electroporation. Overall, successful electroporation as verified by evidence of clear somatic filling with immediate inspection of the recorded cell under epi-fluorescence ($N = 8$) was associated with complete filling of the dendritic arbor when the tissue was inspected after histological processing (7 of 8 neurons). Individual stained cells were easily re-identified under epi-fluorescent illumination after moving to a different

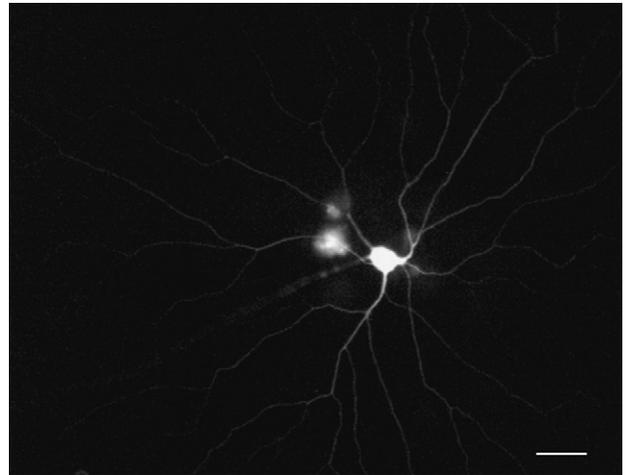


Fig. 4. An OFF α retinal ganglion cell, whose response is shown in Fig. 3, taken within 5 min after electroporation with Alexa Fluoro 568. The cell axon is out of the focus plane, and runs down towards the left from the cell body. Scale bar 50 μm .

location in the retinal piece. Figs. 1 and 2 illustrate two ON ganglion cells immediately after electroporation with the Alexa Fluor-Ames solution. Fig. 1 shows a cell with both infra-red DIC illumination, and epi-fluorescence illumination. In this case the Alexa Fluor filled pipette was used to clear the vicinity of the recorded cell with positive pressure, leading to significant extracellular accumulation of dye. Despite this non-specific staining, the cell body is distinct, allowing further histological processing. Fig. 2 shows another ganglion cell soma immediately after electroporation under epi-fluorescence illumination. In this case no pressure was applied to the pipette containing the Alexa Fluor, and as a result there is little dye in the extracellular space.

Figs. 3–9 show the combined physiological and anatomical results from two classical mammalian retinal ganglion cell types. Among other functional classifications, retinal ganglion cells may be characterized by their selective response to either an increase (ON response) or decrease (OFF response) in stimulus contrast, or both (ON–OFF response), or by a response which depends on the direction of a moving stimulus (directional selective response). The first example, in Figs. 3–5, is a physiological OFF cell (Vaney et al., 1981) whose morphology resembles the type 1 described previously (Amthor et al., 1983), or likewise the common α ganglion cell morphology (e.g. “G11” in Rockhill et al., 2002). For the second example, Figs. 6 and 7 show a phys-

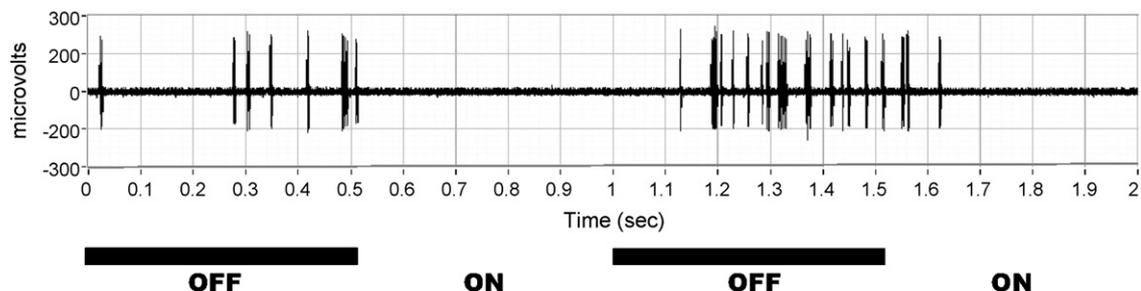


Fig. 3. Average extracellular OFF response of retinal ganglion cell to a full field 100% contrast flashing stimulus (1 Hz temporal frequency, 5 trials). The first transition at $t = 0$ s is from a grey background to black, while the transition at $t = 1$ s is from white to black, yielding a larger OFF response.

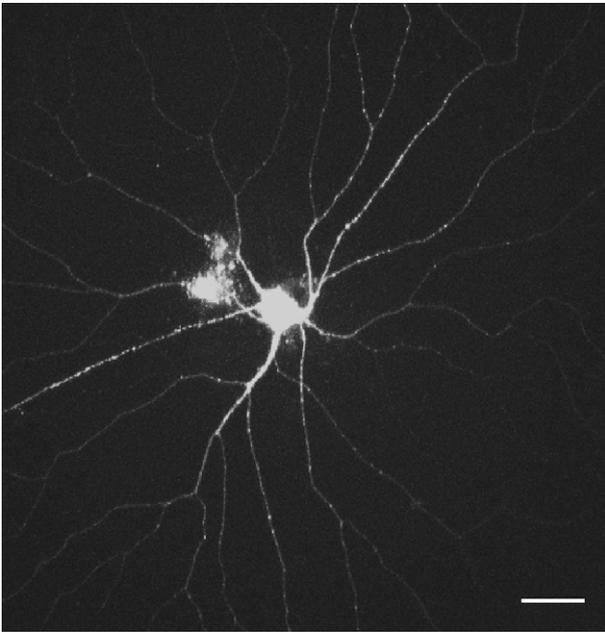


Fig. 5. Confocal image of cell shown in Fig. 4 after fixation. Scale bar 50 μ m.

iological ON–OFF direction selective response (Barlow et al., 1964). Here, the ON–OFF aspect of the cell is indicated by the frequency-doubled response to the moving grating. Figs. 8 and 9 show the anatomy of this cell, with the characteristic highly branched, bi-stratified dendritic tree that is classic for this physiological cell type (Amthor et al., 1984).

4. Discussion

We have demonstrated that the manipulations using a whole-cell patch type electrode typically employed to make single-cell electroporation may be easily adapted to obtain electrophysiological responses from the same cell, simply by using an amplifier that allows both a recording and stimulation mode. As has been reported by other researchers (Rathenberg et al., 2003), successful transfer of the Alexa Fluor required that the pipette tip

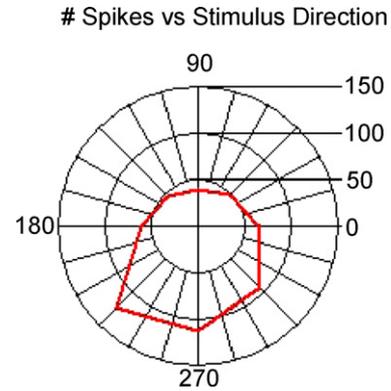


Fig. 7. Polar plot of total spike response (5 trials) as a function of stimulus direction for DS ganglion cell shown in Fig. 6.

be positioned correctly, touching the soma. In our hands, slight negative pressure to bring soma membrane into the tip increased the likelihood of good filling, and at the same time ensured high signal-to-noise ratio recordings of stimulus-evoked action potentials. Occasionally, when the pipette was removed subsequent to the electroporation, the cell nucleus remained attached to the pipette tip, and the cell was destroyed. This seemed to occur more readily when the pipette tip was pushed too vigorously onto the soma membrane, and may be a result of the formation of a true “giga-seal” between the pipette and the cell soma.

To date only intracellular labelling techniques with micro-electrodes (using pressure or iontophoresis), or with patch electrodes in whole-cell mode (using passive diffusion), have allowed simultaneous physiological measurements. An important advantage of the new method over intracellular methods is that in principle the electroporation is less invasive with respect to the target cell. In particular, the whole-cell patch method may disturb the intracellular environment of the recorded neuron to the extent which would compromise the expression of genetic markers.

This method has at least two applications. First, it may be applied to establish the basic structure-function relationship

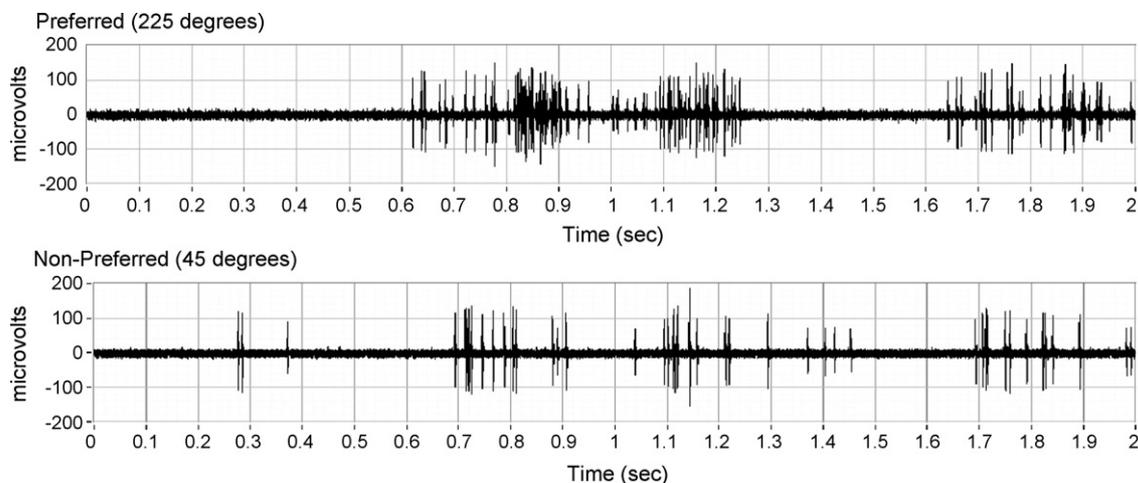


Fig. 6. Average extracellular ON–OFF directional selective (DS) response to two cycles of moving grating (1 Hz temporal frequency, 5 trials), for the preferred and non-preferred stimulus directions.

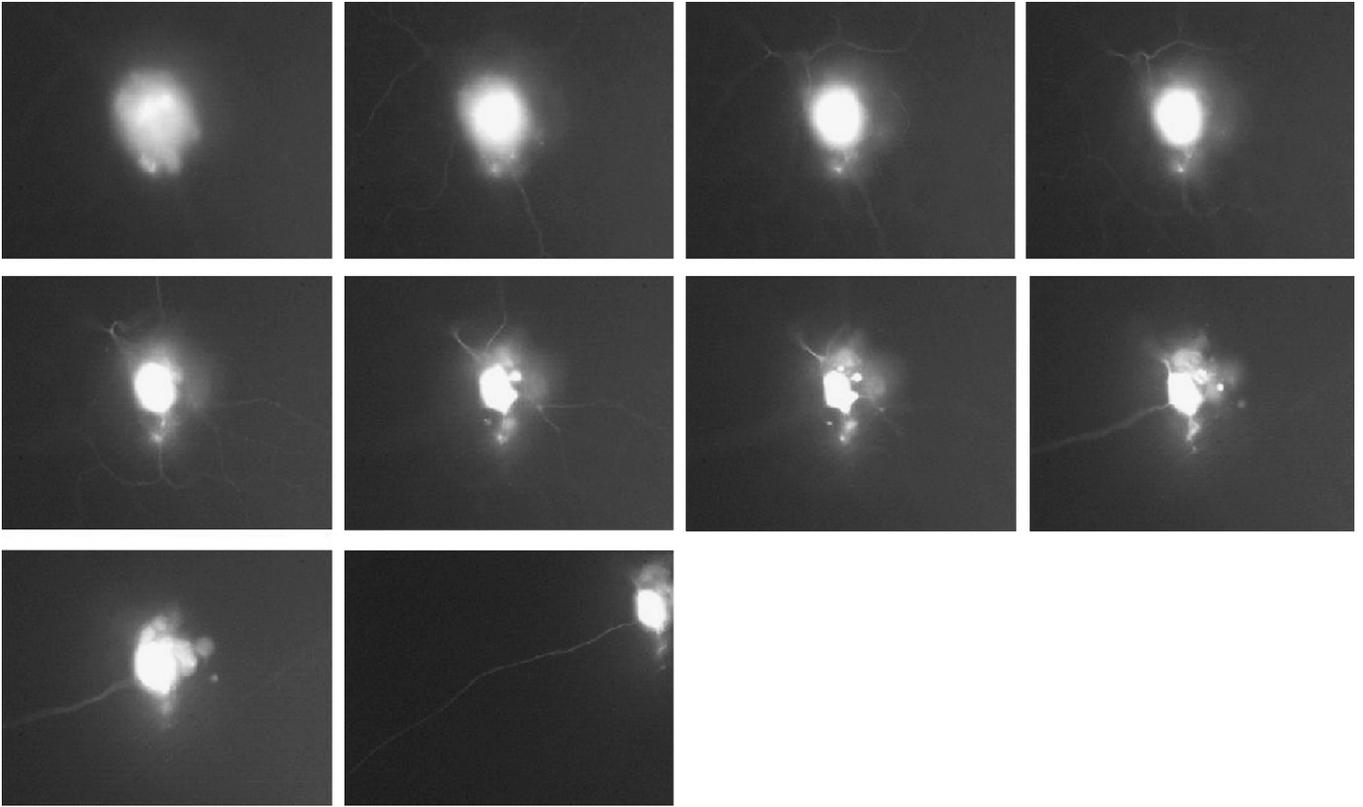


Fig. 8. Depth views of ON-OFF DS ganglion cell whose response is shown in Figs. 6 and 7, immediately after electroporation with Alexa Fluor 568.

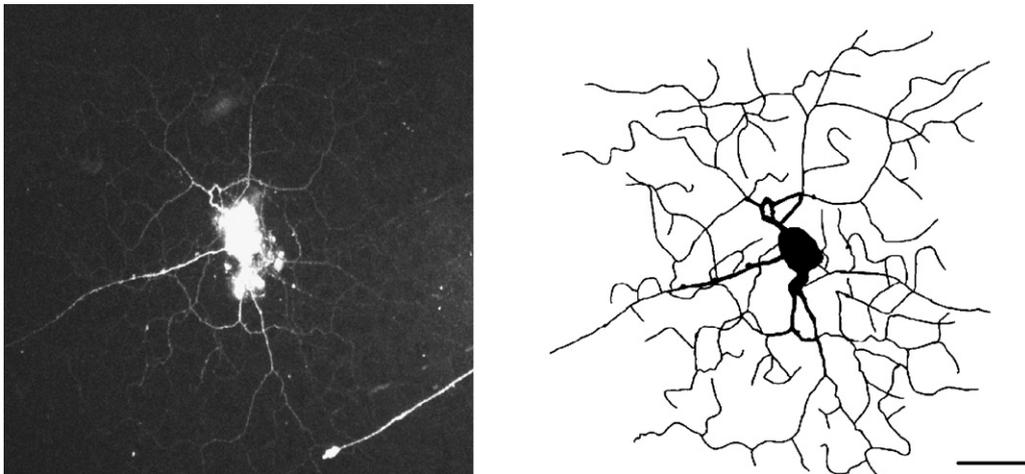


Fig. 9. Left: confocal microscope image of ON-OFF DS cell in Fig. 8 after fixation. The axon in the lower right is from a previously recorded and filled ganglion cell that is approximately 800 μm from the DS cell. Right: tracing of dendritic tree made from the confocal image shown on the left. The preferred direction of movement for this cell was 225° (down and to the left). Scale bar 50 μm .

between different neuron types in retina as well as other areas in which the preparation may be subject to functional stimuli. Second, by incorporating genetic markers in the pipette solution, additional knowledge of the biochemical signature of a single neuron, along with its structure and function, may be obtained. The method described here should also work in vivo, for example targeting cortical neurons. In this case, it will be necessary to infer the physical proximity of the electrode tip from small changes in the electrode resistance, in the same manner as done

with “blind-patch” recordings in vitro (Blanton et al., 1989) and in vivo (Pei et al., 1991). Note that in this case, for retinal recordings, the preliminary step of removing the ILM with a separate surgery electrode would not be required.

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