Video Article

Immunohistochemical and Calcium Imaging Methods in Wholemount Rat Retina

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Abstract

In this paper we describe the tools, reagents, and the practical steps that are needed for: 1) successful preparation of wholemount retinas for immunohistochemistry and, 2) calcium imaging for the study of voltage gated calcium channel (VGCC) mediated calcium signaling in retinal ganglion cells. The calcium imaging method we describe circumvents issues concerning non-specific loading of displaced amacrine cells in the ganglion cell layer.

Video Link

The video component of this article can be found at https://www.jove.com/video/51396/

Introduction

Retinal ganglion cells (RGCs) express L-, N-, P/Q- and T-type VGCC as determined through pharmacological blockade of these components of the whole cell Ca channel current[1,2]. VGCC are transmembrane multimeric proteins that are involved in transmitter release, gene transcription, cell regulation and synaptic plasticity[3,4,5]. Functional VGCCs are made up of at least three distinct classes of subunits: large, transmembrane α_1 pore forming subunits that establish the channel's biophysical and pharmacological properties, primarily extracellular auxiliary $\alpha_2\delta$ subunits and intracellular β subunits. The latter two form heteromeric complexes with different α_1 subunits and alter the gating kinetics and trafficking of the channels to the plasma membrane[6].

Over recent decades, many techniques have been employed to study protein expression, such as immunohistochemistry, enzyme-linked immunosorbent assay, western analysis and flow cytometry. These techniques require the use of specific antibodies for the detection of a given protein of interest and provide powerful tools for the localization and distribution of specific proteins in different tissues. Techniques used to detect and quantify mRNA expression levels of a particular protein such as northern blot analysis, RT-PCR, real-time quantitative RT-PCR, *in situ* hybridization, cDNA microarrays and ribonuclease protection assay provide an alternative approach when antibodies are not readily available or if expression levels of a particular protein are low[7]. However, one limitation to the use of such molecular techniques is the required identification of the gene sequence.

To localize proteins in the retina, immunohistochemistry can be performed on wholemount retinas. Due to the accessibility of the RGCs, the wholemount preparation provides an excellent platform to study the localization of specific proteins to the RGC somata and their axons.

In addition to their localization, some functional properties of the VGCCs in RGCs can be demonstrated through the use of calcium imaging techniques. We describe a calcium imaging protocol to selectively label RGCs with a calcium indicator dye to measure intracellular calcium dynamics. The contribution of different VGCCs to the calcium signal in different cellular compartments can be isolated with the use of subtype-specific Ca channel blockers.

Perhaps one of the most beneficial aspects of the calcium imaging technique described here is the ability to simultaneously and independently record from multiple RGCs and their axons. Although many physiological techniques, such as whole cell patch clamp recording, provide high temporal resolution recordings of membrane currents, the somatic or axonal source of these currents recorded cannot be discriminated and recordings can only be made from a single neuron at a time. Multielectrode arrays (MEAs) are capable of simultaneously recording spikes from many cells, but can neither detect nor discriminate the activation of, for example, different subtypes of calcium channels. MEAs preferentially record from cells that are located in close proximity to a given electrode[8] and record from cells that generate large spikes[9]. Optical imaging methods provide an alternative strategy to enable the simultaneous and independent recordings of whole populations of cells that can be integrated with the information obtained from single cell microelectrode and patch clamp recording and MEA recording. Although the calcium

imaging techniques described here were employed to study the calcium dynamics of RGCs, patch clamp and MEAs can also be used in parallel to further elucidate the ionic currents and spiking properties of RGCs.

Since displaced amacrine cells make up approximately 60% of the neuronal population in the ganglion cell layer in the mouse retina[10], our goal was to use a loading technique that selectively labels RGCs with a synthetic calcium indicator dye in a wholemount preparation. Although synthetic calcium indicator dyes provide an excellent platform for the study of intracellular calcium dynamics, its widespread use has been hindered by the inability to effectively load specific populations of neurons within a given network. Many techniques such as bulk loading[11] and electroporation[8,12] have been performed to load entire populations of cells, however, such techniques do not discriminate between specific cell types. Genetically encoded calcium indicators provide the ability to selectively label specific populations of cells, however, such methods require the generation of transgenic animals[13]. Our technique describes a method to selectively label RGCs in the wholemount preparation via optic nerve stump injection of a calcium indicator dye.

Taken together, the structural and physiological techniques outlined in this article provide a platform to study the localization and contribution of the VGCCs to the calcium signal in RGCs and their axons.

Protocol

All experiments were carried out in accordance with the guidelines for the welfare of experimental animals issued by the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals and the University of California-Los Angeles (UCLA) Animal Research Committee. Male and female adult Sprague-Dawley rats (Charles River Lab, Wilmington, MA) between the age of 3-5 weeks were used.

1. Animal and Tissue Preparation for Wholemount Retina and Immunohistochemistry Protocol

1. Prepare the following materials and tools: A dissecting microscope, 2 forceps with very fine tips, scissors, cellulose filter paper, plastic pipette and a microscope slide.
2. Euthanize the rat in a closed chamber by deeply anesthetizing the animal with 1-3% isoflurane followed by decapitation with a guillotine. Remove the eyes with a pair of iris scissors and place in a Petri dish containing the extracellular solution. Hibernate A, Ames Medium or physiological solutions are recommended.
3. [Optional step if backfilling of RGCs with Fluo-4 is desired]. Perform the Fluo-4 labeling as described in the steps 2.3-2.4.
4. Using the dissecting microscope (light intensity: 1.6 x 10^8 photons/mm^2#sec), remove the cornea by cutting off the front of the eye ball. Remove the lens and vitreous from the inner retinal surface with forceps. To remove the vitreous, stabilize the eyeball by holding the sclera firmly with one forcep. Gently peel the vitreous base toward the center of the retina with the other forcep. The preparation at this stage is called the eyecup, which is used for cryosections. More details about cryosections can be found in references [14,15].
5. Remove the retina from the eyecup. Then, make four cuts to allow the retina to lay flat. Gently mount the retina on a microscope slide with the photoreceptor layer up. Add one drop of solution to the retina and put cellulose filter paper on the retina. Once the retina attaches to the filter paper, place the retina back in solution. If needed, flatten the retina with a brush or forceps.
6. Place the wholemounted retinas into 4% PFA for 10-15 min for fixation.
7. Wash the wholemounted retinas three times for 30 min in 0.1 M phosphate buffer (PB), pH 7.4. Block the retinas with 5% normal goat serum or donkey serum in 0.1 PB containing 0.3% Triton-X 100 and 0.1% NaN_3 at 4 °C overnight. We recommend a final volume of 500 µl for all the steps to save blocking solutions and antibodies.
8. Next day, incubate the retinae wholemounts with the primary antibody 5-7 days at 4 °C. Optimal dilutions must be determined by the user.
9. Wash the retinas 3 x 30 min in PB 0.1 M and incubate overnight at 4 °C in the corresponding fluorophore-conjugated secondary antibody that is directed against the species of the primary antibody (concentration 1:1,000).
10. After three final washes of 30 min each with PB, mount the retinas in mounting medium. Seal the coverslips with nail polish for prolonged storage. Store samples at 4 °C and protect from light.

2. Labeling of Ganglion Cells and Axons in Rat Wholemount Retinas with a Calcium Indicator Dye

1. Prepare 1,000 ml of mammalian Ringer's containing (in mM) 125 NaCl, 3 KCl, 2 $CaCl_2$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 25 $NaHCO_3$, and 10 glucose bubbled with 95% O_2/ 5% CO_2.
2. Perform the dissection as described in the steps 1.2-1.4.
3. To load retinal ganglion cells (RGCs) and their axons with a calcium indicator dye, inject 0.5 µl of Fluo-4 pentapotassium salt (40 mM stock in H_2O) into the optic nerve stump approximately 1 mm posterior to the eyeball using a syringe. To measure dynamic increases in ganglion cell $[Ca^{2+}]_i$ an indicator with high affinity, such as Fluo-4 with its K_d of 345 nM, is optimal. Fluo-4 offers the additional benefit of having a very high quantum efficiency resulting in an emission increase of >100 fold when bound to calcium.
4. Place the eyecup in mammalian Ringer's bubbled with 95% O_2/ 5% CO_2 for 1 hr at room temperature in the dark.

3. Calcium Imaging Protocol for Retinal Ganglion Cells and Axons.

1. Remove the eye, lens and vitreous as described in steps 1.1-1.4. Isolate the retina from the eyecup and divide into quadrants. Mount one quadrant ganglion cell side up on a glass slide and use a harp slice grid to stabilize it. Keep the other pieces dark-adapted in mammalian Ringer's bubbled with 95% O_2/ 5% CO_2 on ice for later use.
2. Superfuse the recording chamber with mammalian Ringer's solution and keep the solution bubbling continuously with 95% O_2/5% CO_2.

3. Image the tissue under the microscope. Conduct experiments at room temperature (~23 °C) or achieve physiological temperatures by warming the stage, heating the water bath under the specimen or applying warm air to the surface of the specimen.
   Note: It is important to note that many cellular functions are regulated by temperature, which plays a key role in maintaining intracellular homeostasis[16]. However, introduction of warming measures may increase the rate of photobleaching, evaporation of the medium and focus drift caused by thermal expansion[17]. Use of room temperature decreases intracellular compartmentalization of Fluo-4[18].
4. Perform a control of two paired high K^+ pulses in the absence of drugs. Depolarize RGCs and RGC axons by raising the $[K^+]_o$ from 3 mM to 60 mM for 33 sec during each pulse to activate VGCCs with an eight channel gravity driven superfusion system. Reduce Na^+ by 57 mM to maintain isosmolarity in the elevated K^+ solution.
5. Assess the contribution of different VGCCs to the calcium signal with the use of general or specific Ca channel blockers. For example, the reduction in calcium signal following the administration of a non-specific calcium channel blocker, cobalt, provided a semi-quantitative measure of the contribution of the VGCCs to the high K^+-evoked calcium signal.
6. Acquire fluorescence intensity values by placing a region of interest (ROI) around the RGCs of interest (**Figure 2**).
7. Normalize the change in fluorescence intensity produced by the second high K^+ peak to the change produced by the first high K^+ peak. Then, for testing of specific calcium channel blockers, apply drugs only during the second high K^+ pulse of a pair and normalize this peak against the first peak value. To measure the affect of the drug on the high K^+ peak, divide the amplitude of the second K^+ peak by that of the first. Analysis should be performed on these values in respect to their matching controls derived from paired high K^+ peaks measured in the absence of drug.
8. Acquire images at 5 sec intervals. To avoid photo-bleaching, keep the laser excitation as low as possible. Provide excitation by the 488 nm line of the argon laser, while the photomultiplier tube collects the fluorescent emission through a 505 nm LP filter.

Representative Results

Immunolabeling with specific antibodies provides a platform to study the localization of particular proteins of interest in the retina and the calcium imaging technique permits the study of the contribution of the VGCCs to the calcium dynamics in the retinal ganglion cells and their axons.

By using an antibody against the ganglion cell protein RBPMS (RNA binding protein with multiple splicing) that selectively labels RGCs[19], we were able to show that the Fluo-4 labeling is limited to ganglion cells (**Figure 1**). Stump-injection does not lead to the labeling of all ganglion cells as might be anticipated from this technique.

A rabbit polyclonal antibody was generated against the N-terminus of the RBPMS polypeptide ($RBPMS_{4-24}$), GGKAEKENTPSEANLQEEEVR, by a commercial vendor (**Table 1**). RBPMS is highly conserved among mammals and the polypeptide sequence used for immunization is identical in mouse, rat, monkey and human (NCBI Protein Bank, http://www.ncbi.nlm.nih.gov/protein). Rabbit sera was collected following immunization and affinity purified using a RBPMS polypeptide affinity column. The affinity purified antibody was shown to immunostain ganglion cells in mouse and rat retina. To evaluate the specificity of the RBPMS immunostaining, a preabsorption control was performed with the rabbit antibody. Briefly, the RBPMS antibody was diluted in 0.1 M PB containing 0.5% Triton X-100 and mixed with the RBPMS polypeptide at a final concentration of 1 µg/ml for 2 hr at RT. No RBPMS immunostaining was present in tissue sections incubated with the preabsorbed rabbit antibodies to RBPMS and processed by standard immunohistochemical techniques.

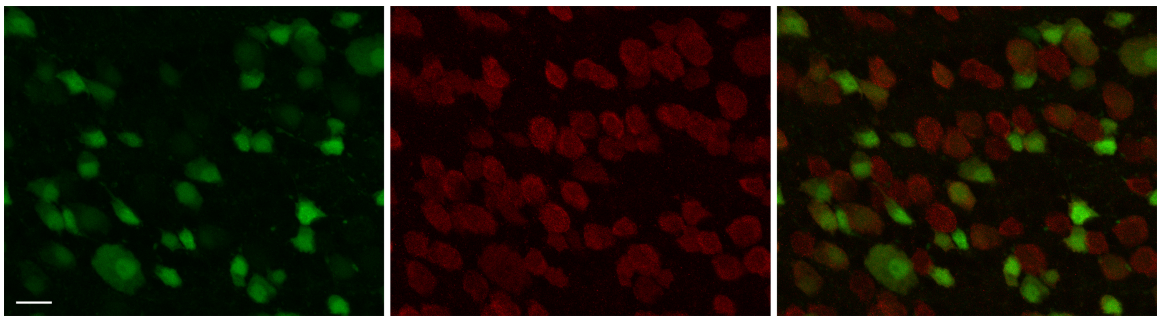

Figure 1: Post-hoc immunostaining with antibodies against the RGC protein RBPMS (red) to show Fluo-4 (green) localization to the ganglion cells. An Alexa 568 goat anti-rabbit secondary antibody was used. Scale bar: 20 mm.

Calcium imaging techniques are used to study the contribution of VGCCs to the calcium signal in the RGCs and their axons. Fluorescent intensity values were acquired by placing ROIs on the RGCs of interest (**Figure 2**).

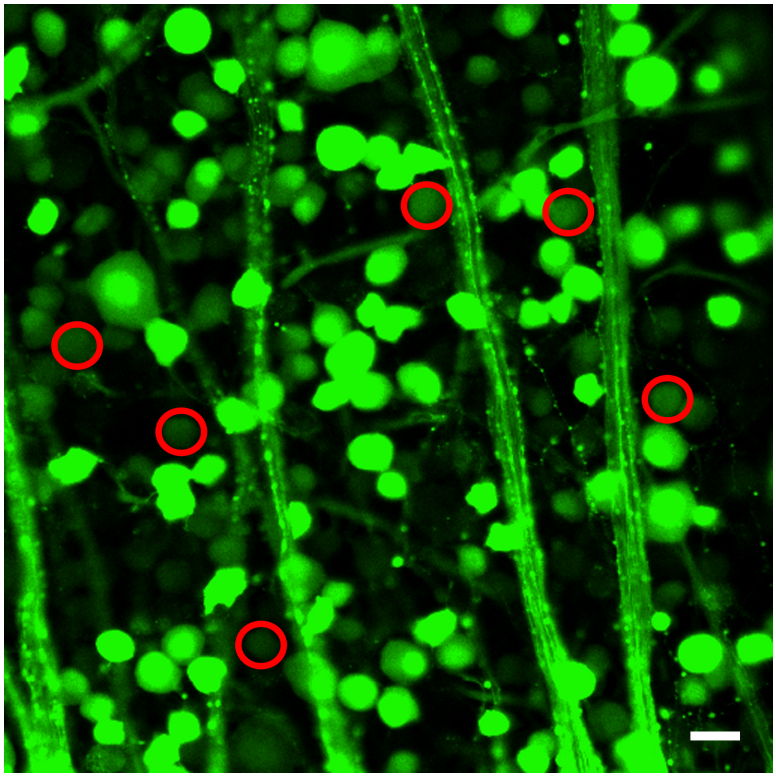

Figure 2: In order to acquire fluorescent intensity values, ROIs were placed on the ganglion cell somata and/or axons. Scale bar: 50 mm.

Fluo-4 pentapotassium salt was used to label RGCs and their axons (**Figure 3**).

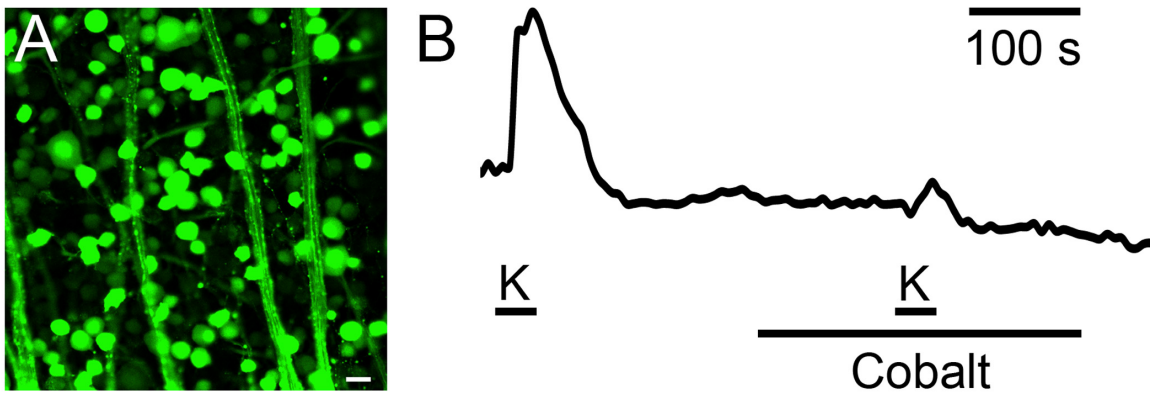

Figure 3: VGCC subtypes that contribute to calcium signaling in ganglion cell somata and their axons. (A) Confocal image of a wholemount retina at baseline levels. RGCs and RGC axons can be seen labeled with fluo-4. **(B)** Fluorescent signals from a single RGC somata demonstrating the increase in mean fluorescence intensity elicited by paired pulses of high-K^+ (60 mM) bathing solution and the subsequent reduction in fluorescence in the presence of cobalt during the second pulse. Scale bar for A: 20 mm.

The Zeiss LM5 Pascal system was set to capture images at a frame rate of 5 sec and each full frame scan took 1.57 sec at the settings used. A collection area of 318 µm x 318 µm was included in the scanned region of 512 x 512 pixels giving an area of 0.385 μm^2 per pixel. These settings may be varied depending on the resolution desired. The system uses a 25 mW Argon laser for the 488 nm excitation. We used this line at 3% power. Saturation was minimized by first selecting the lowest concentration of the calcium indicator dye that yielded the robust responses to the depolarizing stimulus and then by reducing output gain. Reduction of laser power helped to avoid bleaching. Indicator concentration was not measured. Calibration of non-ratiometric imaging is possible with the use of calcium ionophores, but the backfilling technique may work with ratiometric dyes as well. To determine the optimal indicator concentration, we compared the depolarization-evoked change in fluorescence with stump-injected concentrations of Fluo-4 ranging from 1-40 mM. Circular and oval ROIs were drawn around RGCs and their axons, respectively that exhibited stable fluorescence intensity. RGCs and axons that drifted out of focus due to movement caused by superfusion were not selected.

Discussion

In this article, we have described two different techniques: 1) Immunohistochemistry to show Fluo-4 localization to the ganglion cells in retinal wholemounts, and 2) Calcium imaging to analyze calcium dynamics in retinal ganglion cells and their axons.

Immunohistochemistry using wholemounts has been used to reveal the localization of proteins in the rodent retina[20-23]. However, there are a few limitations. The quality of the staining relies heavily on the ability of the antibody to recognize its respective antigen and its ability to penetrate the retinal tissue to the particular layer of interest. These issues can be improved by increasing the incubation period in the primary antibody by up to seven days, increasing the concentration of Triton-X and/or incubating the retina without the filter paper to allow the diffusion of the antibody through the ganglion cell layer and the photoreceptors. Another limitation is the duration and concentration of fixation. Some antibodies work better when the tissue is fixed for one hr, while other antibodies require a shorter fixation period. Likewise, while certain antibodies work best in 4% PFA fixation, others work better at reduced concentrations. We recommend that the user determine the optimal duration and concentration of fixation, the duration of incubation, the concentration of Triton-X, as well as the concentration of the primary antibody for optimal results.

Calcium imaging was used to study the calcium dynamics in retinal ganglion cells and their axons. Superfusion of VGCC blocking agents allowed assessment of the relative blocking efficacy of the drugs and the presence of different Ca channel subtypes. In the event that RGCs and axons do not respond to high K^+, try reducing the concentration of the calcium indicator dye, as high concentrations can lead to saturation. Incomplete removal of the vitreous may also act as a barrier, preventing high K^+ from reaching the RGCs and their axons. In addition, failure to properly mount the harp on top of the retina can cause movement, resulting in an inability to acquire fluorescence intensity values. If the retina continues to move, carefully remove the harp, dry the area surrounding the retina and re-mount the harp by adding more grease to its perimeter. Complete removal of the vitreous and minimal movement of the retinal wholemount is crucial to the success of the experiment.

Agonists of the calcium-permeable glutamate receptors expressed by RGCs such as *N-methyl-D-aspartate (*NMDA),[24,25] *α*-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors and kainate-type ionotropic glutamate receptors[26-29], can be used to evoke direct entry of calcium as well as provide a depolarizing stimulus that activates VGCCs. Utilization of such agonists may lead to a non-uniform response due to the differential expression of the glutamate receptor subtypes to the different types of RGCs. Two-photon microscopy can also be utilized to investigate light-evoked calcium signals in RGCs[30] and their dendrites[31].

Selective labeling of RGCs was achieved due to the membrane impermeant properties of the Fluo-4 pentapotassium salt calcium indicator dye. Dextran-conjugated calcium indicator dyes have also been used to selectively label RGCs and their axons via intraretinal injections in rabbit[32] and rat[33]. In both studies, selective labeling of RGCs and their axons was achieved via intraretinal injections of the dextran-conjugated calcium indicator dye, followed by a 2 hr incubation in rabbit[32] or a 7 hr incubation in rat[33]. Despite providing an adequate incubation period to ensure uniform labeling in the retina, such methods result in increased labeling of RGCs and their axons nearest the injection site compared to sparse labeling in regions distal to the injection site. As discussed in Baldrige (1996), non-uniform labeling at the injection site has been attributed to diffusional labeling of the soma as a result of dye uptake at the exposed (cut) dendrite, while labeling in distal regions is likely due to retrograde transport from uptake of the dye at the exposed axon[32]. Our technique provides improved methods to increase uniform labeling of RGCs and their axons and to minimize the required incubation period.

The present dye loading method provides an alternative strategy to conventional techniques such as bulk loading and electroporation that result in non-specific loading of displaced amacrine cells in the ganglion cell layer. With the availability of transgenic mouse lines expressing tdTomato or EGFP under the control of promoters that are in specific ganglion cell populations[34-38], future applications of this technique may include calcium imaging studies of specific retinal ganglion cell subtypes.

Disclosures

The authors have no disclosures.

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