Video Article

Simultaneous *ex vivo* Functional Testing of Two Retinas by *in vivo* Electroretinogram System

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Abstract

An *In vivo* electroretinogram (ERG) signal is composed of several overlapping components originating from different retinal cell types, as well as noise from extra-retinal sources. *Ex vivo* ERG provides an efficient method to dissect the function of retinal cells directly from an intact isolated retina of animals or donor eyes. In addition, *ex vivo* ERG can be used to test the efficacy and safety of potential therapeutic agents on retina tissue from animals or humans. We show here how commercially available *in vivo* ERG systems can be used to conduct *ex vivo* ERG recordings from isolated mouse retinas. We combine the light stimulation, electronic and heating units of a standard *in vivo* system with custom-designed specimen holder, gravity-controlled perfusion system and electromagnetic noise shielding to record low-noise *ex vivo* ERG signals simultaneously from two retinas with the acquisition software included in commercial *in vivo* systems. Further, we demonstrate how to use this method in combination with pharmacological treatments that remove specific ERG components in order to dissect the function of certain retinal cell types.

Video Link

The video component of this article can be found at https://www.jove.com/video/52855/

Introduction

Electroretinogram (ERG) is a well-established technique that can be used to record the electrical activity of the retina triggered by light. The ERG signal is generated mainly by voltage changes caused by radial currents (along the axis of photoreceptors and bipolar cells) flowing in the resistive extracellular space of the retina. The first ERG signal was recorded in 1865 by Holmgren from the surface of a fish eye[1]. Einthoven and Jolly 1908[2] divided the ERG response to the onset of light into three different waves, called a-, b-, and c-waves, that are now known to reflect mainly the activity of photoreceptors, ON bipolar cells, and pigment epithelium cells, respectively[3-8]. ERG can be recorded from the eyes of anesthetized animals or humans (*in vivo*), from isolated eye preparation[9], across isolated intact retina (*ex vivo*)[3,10-15] or across specific retina layers with microelectrodes (local ERG)[4,16]. Of these, *in vivo* ERG is currently the most widely used method to assess retinal function. It is a noninvasive technique that can be used for diagnostic purposes or to follow the progression of retinal diseases in animals or patients. However, *in vivo* ERG recordings produce a complicated signal with several overlapping components, often contaminated by extraocular physiological noise (*e.g.,* breathing and cardiac activity).

Local ERG can be used to record the signal across specific layers of the retina but it is the most invasive and has the lowest signal-to-noise ratio (SNR) as compared to the other ERG recording configurations. Local ERG is also technically demanding and requires expensive equipment (*e.g.,* microscope and micromanipulators). Transretinal ERG from the intact, isolated retina (*ex vivo* ERG) offers a compromise between *in vivo* and local ERG methods allowing stable and high SNR recordings from intact retinas of animals or humans[17]. Recently, this method has been used successfully to study rod and cone photoreceptor function in mammalian, primate and human retinas[18-20]. In addition, due to absence of pigment epithelium in the *ex vivo* retina, the positive c-wave component of the ERG signal is removed and a prominent negative slow PIII component is revealed in the *ex vivo* recordings. The slow PIII component has been shown to originate from the activity of Müller glia cells in the retina[21-23]. Thus, *ex vivo* ERG method could also be used to study Müller cells in the intact retina. Several studies have also shown that *ex vivo* ERG recordings could be used to measure concentration of pharmacological agents around the retina[24] and test the safety and efficacy of drugs[25-27].

Multiple commercial *in vivo* systems are available and used in many laboratories that do not necessarily have extensive electrophysiology background. In contrast, *ex vivo* devices have not been available until recently[17] and as a result only very few laboratories are currently taking advantage of this powerful technique. It would be beneficial to make *ex vivo* ERG recordings available to more laboratories in order to advance our knowledge about retinal physiology and pathology, and to develop new therapies for blinding diseases. We demonstrate here a simple and affordable *ex vivo* ERG device[17] and show how it can be used in combination with several commercially available *in vivo* ERG systems to record rod- and cone-mediated signaling (a- and b-waves) and the function of Müller cells (slow PIII) from intact wild-type mouse retinas.

Protocol

All experimental protocols were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the institutional Animal Studies Committee at Washington University.

1. Setting Up Perfusion and Specimen Holder

1. Prepare solution for retina perfusion fresh on the day of the experiment. Use distilled and deionized water. Use one of the following three solutions.
    1. Prepare Bicarbonate-containing Ames' solution (1 L): 1 bottle of Ames' media and 1.9 g of $NaHCO_3$,
    2. Prepare Locke's solution (in mM): 112.5 NaCl, 3.6 KCl, 2.4 $MgCl_2$, 1.2 $CaCl_2$, 10.0 HEPES, 20.0 $NaHCO_3$, 3.0 Na succinate, 0.5 Na glutamate, 0.02 EDTA, and 10.0 glucose, 0.1% MEM vitamins and amino acids
    3. Prepare HEPES-buffered Ringer solution (in mM): 133.3 NaCl, 3.3 KCl, 2.0 $MgCl_2$, 1.0 $CaCl_2$, 10.0 glucose, 0.01 EDTA, 12.0 HEPES, pH adjusted to 7.5 with ~5.8 ml of 1 M NaOH, add 0.72 g/L Leibovitz culture medium L-15. Use 20 - 50 µM DL-AP4 and 50 – 100 µM $BaCl_2$ to isolate the photoreceptor response with any perfusion media.

2. Prepare solution for electrodes[28] (in mM): 140.0 NaCl, 3.6 KCl, 2.4 $MgCl_2$, 1.2 $CaCl_2$, 3 HEPES, 0.01 EDTA and adjust pH to 7.4 – 7.5 with NaOH. Electrode solution can be stored at RT for several months.
3. Prepare and test the specimen holder.
    1. Glue black/grey filter paper on top of the domes of the specimen holder's bottom part (see **Figure 1A**). Spread two-component 5 min epoxy glue carefully around the edges of the flat tops of the domes. If necessary, do it under dissection microscope.
    2. Wait until the glue is almost dry (about 4 min) and press the filter paper on the domes by using a flat item. Glue filter paper at least one day before the experiment. The filter paper can be used multiple times but should be replaced after a month of recordings. Use 70% ethanol to clean domes carefully from any glue residue before installing the replacement paper.
    3. Fill the electrode channels with electrode solution trying to avoid any air bubbles and screw pellet electrodes enclosed with a threaded adapter into the electrode channels (see **Figures 1A** and **B**). Connect the top and bottom pieces of the specimen holder with four screws and fill the perfusion lines with electrode solution.
    4. Measure the resistance and voltage between the leads of each electrode pair using a multimeter (**Figure 1B**). Resistance should be below 100 Ωk and the voltage below 10 mV if the channels are bubble-free and the electrodes are in good conditions.

4. Pour 400 - 700 ml of perfusion media in the glass bottle. Separate another 300 ml to be used in the dissection of the retinas and store it in a fridge. Set the perfusion tubing in the heat exchanger block and place the preheated block on the heating plate (see **Figure 1D**).
5. Enclose the bottle with a cap that has connections to CO_2/O_2 gas (if Ames' or Locke's is used) and for perfusion tubing (see **Figure 1D**). Preheat the bottle with the media to 37 ^oC and place it on the heating plate or on the top of the light stimulation unit in water bath set to 37 – 39 ^oC[17].
    1. Prime the perfusion lines by filling them with perfusion media to initiate the gravity-driven flow.
        1. In LKC system, place the bottle on the top of the stimulator unit[17] to provide a significant gravitational flow that is then adjusted by flow-rate regulators without being affected by the lowering level of the perfusion solution in the bottle during the experiment.
        2. In Ocuscience system, place the perfusion bottle inside the Faraday cage in order to minimize noise and place long perfusion output lines from the specimen holder (see step 2.8) well below the level of the specimen holder (and perfusion bottle) to increase gravitational drive of the solution. Shield these output lines and connect the shield to the amplifier's ground to prevent coupling of the electromagnetic noise to the ERG signal.

6. Adjust the perfusion to 3 – 5 ml/min by using flow-rate regulators. Connect 5% CO_2 / 95% O_2 gas from a cylinder with proper regulator and adjust the flow rate to ensure steady bubbling of the media in the bottle through an air stone.

2. Sample Preparation

1. Assemble clean and sharp dissection instruments including straight-bladed microscissors, one or two 45^o tweezers, razor blade and a rectangular piece of filter paper.
2. Pour about 200 ml of cold perfusion solution in a large Petri dish so that the whole bottom part of the specimen holder (including the domes) can be immersed into the solution. This step becomes important when mounting the retinas on the domes (see step 2.6). Although some solutions are designed to be saturated with carbogen (5% CO_2 / 95% O_2), this is not essential for the dissection purposes and was not done in the experiments described here.
3. For a typical experiment, keep animals in 12/12 hrs dark/light cycle and dark-adapt them for 6 – 12 hrs before the recordings. Euthanize the animal by CO_2 inhalation followed by cervical dislocation under dim red light and do all the following procedures under dim red or IR light (use *e.g.,* red filter in front of the microscope light source).
    1. Pull the eyes out by using tweezers and put them in the media. Place one eye at a time on a small piece of paper (*e.g.,* some regular filter paper) and make a slit approximately on the level of ora serrata while holding the eye with tweezers (this is done outside of the solution here).

4. Cut along the ora serrata (or closer to the equator of the eye) with microscissors and remove the cornea and the lens. Place the eye cup in the cold media in the large Petri dish and repeat the same procedure with the other eye.
5. Cut a small incision from the top of the eye cup towards the optic nerve by keeping the scissors between the retina and sclera in order to keep the retina as intact as possible. Grip the sclera from both sides of the incision by using two tweezers and pull the tweezers away from each other to detach the retina.

1. Cut the optic nerve and try to isolate the retina with a minimum amount of physical contact to the distal surface. RPE will mostly detach automatically from the retina during the dissection process. It is more important to avoid mechanical disturbance of the retina than to perform the dissection quickly, generally retinas can be incubated at least 30 min in the solution without significant effects on the response properties.

6. Mount the retinas on the domes of the specimen holder (**Figure 1C**). Immerse the bottom part of the specimen holder in the Petri dish with the dissected retinas. Slide the retina, photoreceptor side (the convex surface of the isolated retina) upwards, above the dome and lift the specimen holder so that the retina attaches on the filter paper. Repeat the procedure for the other retina.
7. Dry the holder plate carefully to prevent electrical crosstalk between the retinas as well as noise and signal shunting. Specimen holder has O-rings around the bottom part domes to prevent solution spill between the bottom and top parts of the holder as well as to help electrical isolation of the photoreceptor and ganglion cell sides of the individual retinas. Attach the top part of the holder with the four screws (see **Figure 1B**) and fill the perfusion channels with perfusion solution by using a syringe and a needle.
8. Transfer the specimen holder next to the heat exchanger block and connect the input and output perfusion lines to the specimen holder (**Figure 1D**). Connect the electrodes to the ERG amplifier (top electrodes connect to the ground/minus pin in the amplifier) and attach the stimulator/control unit on the specimen holder by using an adaptor or slide the heating pad into the stimulator unit depending on which *in vivo* ERG system is used.

3. Recordings

1. Configure the amplifier and stimulus settings by using the *in vivo* system's software. Set the acquisition frequency to a value between 1 and 10 kHz and low-pass filtering to 300 Hz. Do not use high-pass filtering. Use 60 Hz (or 50 Hz in Europe) notch filter if necessary.
2. Record baseline without light stimulation and with dim stimulation (*e.g.*, green light of -35 dB or 0.3 mCd sm^{-2}) to test for good electrical connection between the electrodes and retina sample. Wait 10 – 20 min before starting the data collection so that retinal temperature and function reach a stable state.
    1. Choose stimulus parameters according to any specific experiment and start data collection. *E.g.,* use green light from -40 up to 0 dB or from 0.1 up to 1,000 mCd sm^{-2} for a scotopic response family. Use about 2 to 3 log-units brighter flashes in photopic recordings where rods have to be suppressed by background light (3 – 30 Cdm^{-2}) or a probe flash (about 1.3 Cd sm^{-2} = 1 dB, see **Figure 3**).
    2. However, remember that the exact light intensity values might be somewhat dependent on your system calibration and experimental conditions. As a rule of thumb, scale intensities down by 5 – 10-fold as compared to *in vivo* recordings when using green light[17]. A black cover with apertures above the retinas (included in the commercial adapter system) can be used to reduce light scattering in the specimen holder and facilitate homogenous and equal stimulation of both retinas by the Ganzfeld sphere of the commercial *in vivo* systems.
    NOTE: For recordings from dark-adapted retina, the intensities of test flashes used in a typical experiment bleach only a negligible fraction of the pigment so that the lack of RPE-driven regeneration is not an issue. In addition, it has been previously shown that cone pigment regeneration can still take place in the isolated retina via the Müller cell visual cycle[20].

3. As experiments last typically from 30 min up to several hours, monitor baseline drift, noise level and response stability during the experiment as changes in these might indicate technical problems.
NOTE: Increased noise or decreased response amplitudes may indicate air bubbles in the perfusion or electrode channels, too low or high temperature, solution leak/spill or displacement of the retina.

4. Cleaning

1. Detach the specimen holder from the perfusion lines, open it and flush the retinas from the filter paper. Remove the electrodes and rinse them with distilled water (do not use ethanol). Clean the specimen holder (including the perfusion channels) with ethanol and/or distilled water. Flush perfusion tubing carefully with >70% ethanol.
2. Rinse the perfusion bottle with distilled water (do not use detergents). Ethanol can be also used to clean the bottle.

Representative Results

We recorded flash responses from dark-adapted wild-type (WT) C57BL/6 mouse retinas by following the experimental protocols described above and illustrated in **Figure 1** by using different standard perfusion solutions (**Figure 2**). The response waveforms and kinetics as well as sensitivity of rod photoreceptors appeared similar in Ames' and Locke's media (**Figure 2A** and **B**). On the other hand, under HEPES-buffered Ringer solution (no bicarbonate or 5% CO_2 / 95% O_2) the response amplitudes were significantly smaller. We also found that in these conditions b-wave stability was compromised. Adding 40 µM APB (DL-AP4) removed positive b-wave efficiently in all three media (**Figures 2D-F**). Removal of the b-wave revealed a large slow negative wave (**Figure 2D**) that has been attributed to Müller cell activity[21]. Adding 100 µM of barium abolished this component, revealing the photoreceptor response of the *ex vivo* ERG signal (**Figures 2D-F**). We could record up to 1 mV saturated photoreceptor responses in Ames' and Locke's whereas maximum responses were typically around 200 µV under Ringer perfusion.

Cone photoreceptor responses have been isolated previously by so-called double flash technique where a bright probe flash saturating the rods is followed by a test flash at a time when cones have restored their dark-adapted state but rods remain saturated[19,29]. Here we isolated cone-mediated ERG responses (containing both a- and b-wave) in Ames' media supplemented with 100 µM of barium but not DL-AP4 by using double flash technique (**Figure 3**). Barium was used to remove the slow glial component that appeared to make the late part of the responses more variable especially during repetitive use of bright flashes. We used a constant probe flash to saturate rods and variable test flashes 300 msec after the probe flash to elicit cone responses. A cone response family obtained by subtracting probe flash response from the 'probe + test flash' responses is shown in **Figure 3B**.

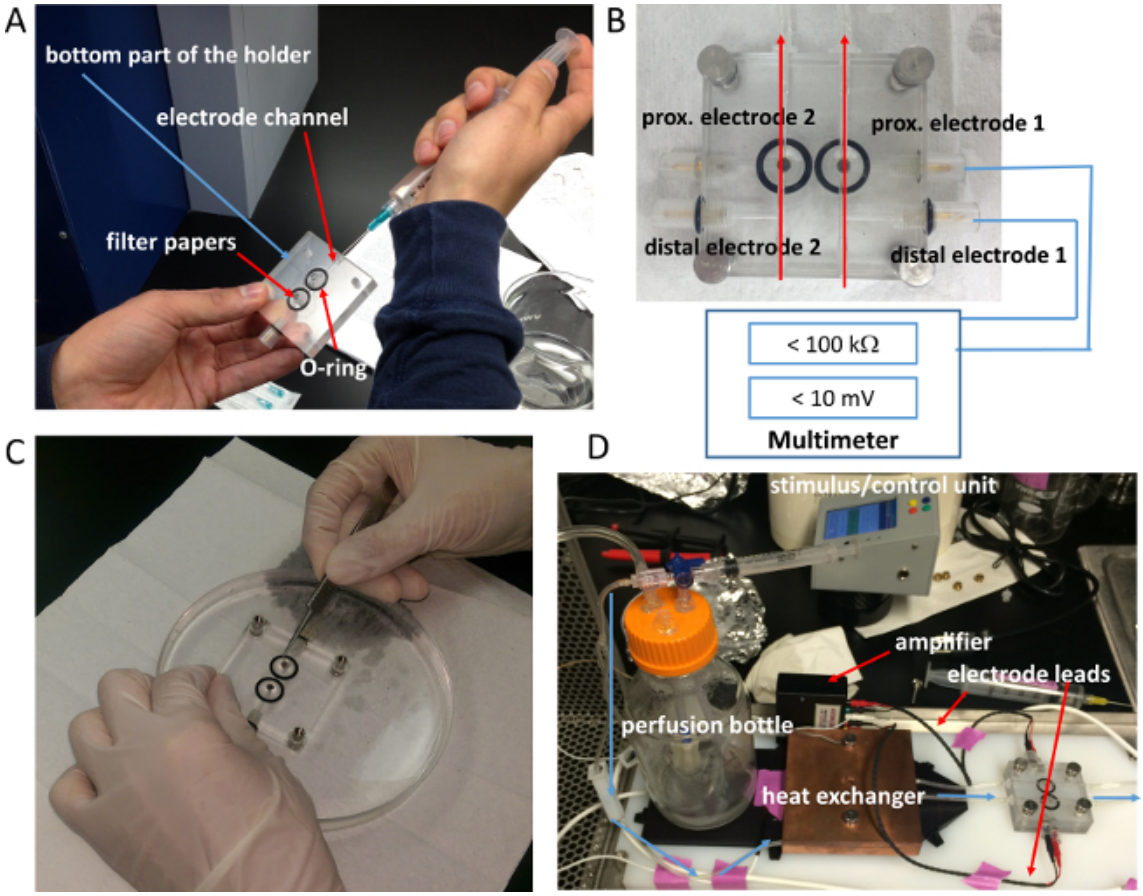

Figure 1. Use of *ex vivo* ERG specimen holder. (A) Filling of electrode channels and mounting of the electrodes. **(B)** Testing of the assembled specimen holder before dissection by measuring the resistance and voltage between the electrode pairs. **(C)** Mounting of the retina on the filter paper in the specimen holder. **(D)** The specimen holder connected to the perfusion lines and ERG amplifier in the commercial ERG system. The perfusion flow path is indicated by blue arrows. Please click here to view a larger version of this figure.

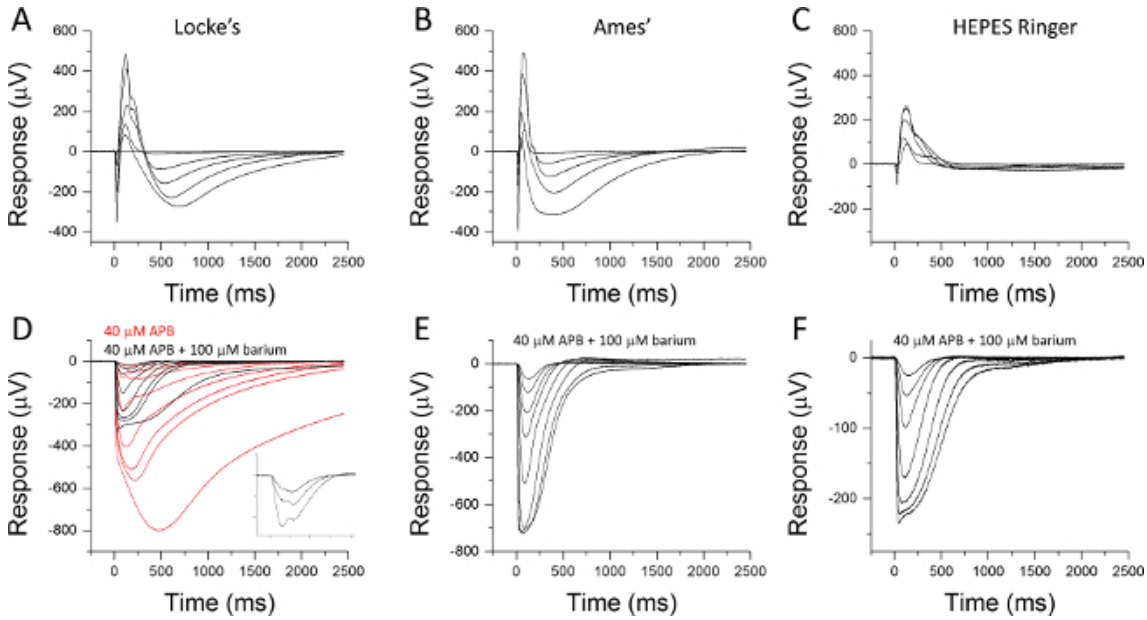

Figure 2. Flash response families recorded from dark-adapted WT mouse retinas perfused with Locke's **(A)**, Ames' **(B)**, and HEPES-buffered Ringer **(C)** medium. The flashes delivered 3, 40, 130, 390 and 1,400 photons µm^{-2} (from -36 to -9 dB or -3.6 to -0.9 log(Cd sm^{-2}) green light (530 nm)). Black traces in **(D-F)** show responses recorded from retinas in **(A-C)** after addition of 40 µM APB and 100 µM barium. Red traces in **(D)** show responses recorded from the retina in **(A)** perfused with Locke's supplemented with 40 µM APB but not barium. The inset shows the responses to the three dimmest flashes in Locke's media containing APB and barium. The flashes ranged from 7 to 14,000 photons µm^{-2} (from -32 to 1 dB green light) in **(D)**, from 3 to 1,400 photons µm^{-2} (from -36 to -9 dB green light) in **(E)**, and from 7 to 1,400 photons µm^{-2} (from -32 to -9 dB green light) in **(F)**. See Vinberg *et al.* 2014[17] and Lyubarsky *et al.* 1999[30] and 2004[31] for details of converting photopic luminous energy given in Cd sm^{-2} to photons µm^{-2}. Please click here to view a larger version of this figure.

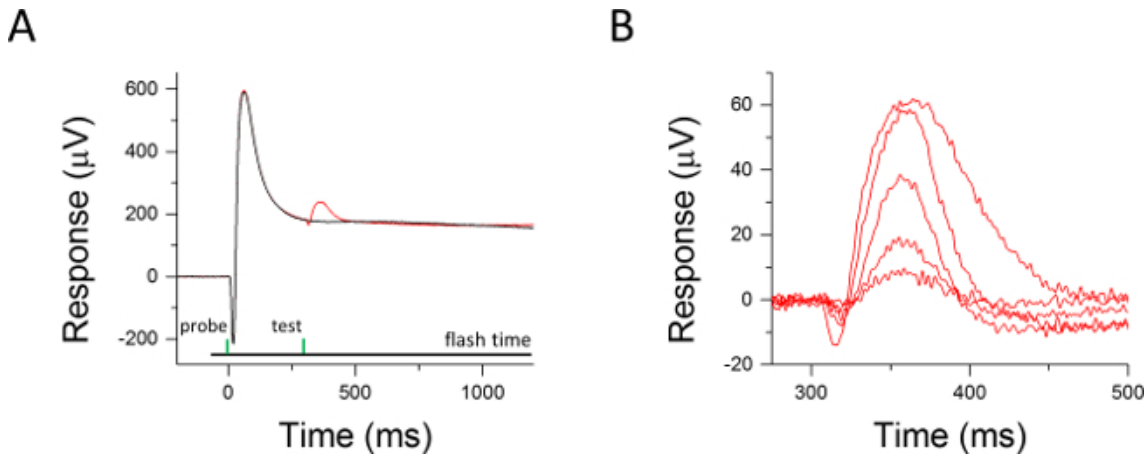

Figure 3. Isolation of cone responses with double flash method in WT mouse. (A) A response to probe flash (14,000 photons µm^{-2} or 1 dB green light, black) and a response to probe flash followed by a test flash (81,000 photons µm^{-2} or 9 dB green light, red). **(B)** Cone flash responses to test flashes ranging from 360 to 81,000 photons µm^{-2} (-14 to 10 dB green light) isolated by subtracting the probe flash response from the "probe + test flash" response. Please click here to view a larger version of this figure.

Discussion

We demonstrate here the critical steps for obtaining high-quality *ex vivo* ERG recordings simultaneously from two isolated mouse retinas by using *in vivo* ERG system components together with an *ex vivo* ERG adapter. In this study we perfused both retinas from the animal with the same solution (either Ames', Locke's or Ringer) but it is also possible to perfuse each retina with a different solution *e.g.,* for drug testing purposes. The most important steps for obtaining high quality data are shielding from electromagnetic noise, careful dissection of the retina, steady and relatively rapid perfusion flow by using an advanced custom-built specimen holder, and performing all sample preparation procedures under dim red (or IR) light. The method described here allows immediate use of both retinas and dual use of an *in vivo* ERG setup to perform *in vivo* and *ex vivo* ERG recordings.

The custom-built *ex vivo* ERG specimen holder recently designed by us[17], and now commercially available, improves SNR by efficient electrical isolation of the proximal and distal parts of the retina and optimizes perfusion flow above the retina (high solution exchange rate). Absence of the slow frequency noise components in *ex vivo* ERG signal allows quantitative analysis even from very small responses. However, we found that *ex vivo* recording is more prone to interference of AC-power line noise (60 Hz in the US; 50 Hz in Europe) than *in vivo* experiments. This noise

coupled to the signal mainly through the perfusion line, and it could be mostly removed by shielding (and grounding) all perfusion components (bottle, tubing) residing outside of the Faraday cage or Ganzfeld sphere. In addition, sometimes 60 Hz noise coupled to the *ex vivo* ERG signal through the heat exchanger and this noise could also be removed by grounding.

We demonstrate how to remove specific ERG signal components by adding pharmacological blockers into the perfusion during the experiment allowing dissection of the function of different cell types in the same retina/experiment with three different physiological perfusion media (**Figure 2**). A recent study showed that the choice of perfusion media affects photoreceptor physiology in single cell recordings[32]. Here we used Ames', Locke's and 'HEPES-Ringer' media to record dark-adapted flash responses in the absence and presence of pharmacological reagents intended to isolate the photoreceptor component of the ERG signal (**Figure 2**). Bicarbonate-buffered solutions gave larger a- and b-wave amplitudes, up to 1 mV. Photoreceptor dim flash responses under Locke's medium with blockers contained complicated recovery waveform (see inset of **Figure 2D**) that was not seen with Ames' or Ringer perfusion. When the use of *ex vivo* ERG becomes adapted by more laboratories it would be helpful to use the same perfusion media and standard methods to isolate different signal components. At this point it seems that the most versatile option is the Ames' medium because it gives stable and large a- and b-wave amplitudes. In addition, the photoreceptor response, isolated pharmacologically in this solution, appears to have a simple waveform reminiscent of that recorded from single photoreceptors (**Figure 2E**). Yet, some open questions remain about the existence of other ERG signal components observed under *in vivo* conditions. For example, in our *ex vivo* recording conditions we did not see prominent oscillatory potentials, 100 – 150 Hz oscillating waves that are typically observed in the rising phase of the b-wave of an *in vivo* ERG response. It is thus possible that the inner retina function in our *ex vivo* conditions was compromised although large *ex vivo* b-waves implicated viable ON bipolar cell function. Future studies should resolve whether modifications in the experimental protocols shown here (dissection, perfusion etc.) would allow us to record oscillatory potentials under *ex vivo* conditions.

Cone function is vital for our vision. However, investigation of cones is hampered by their small size and scarcity especially in the mouse retina[33]. Isolation of cone function is further complicated in mouse ERG recordings because their M-cones and rods have almost identical spectral sensitivities[30]. Standard protocols take advantage of the several log-unit difference between rod and cone sensitivities by using rod-suppressing background light. However, steady background light is known to desensitize rods[34,35] and affect cone function possibly through modulation of gap junctional coupling between rods and cones[36,37]. Thus, it is hard to find a background light intensity that is bright enough to keep rods saturated without affecting cones. Here we demonstrate an alternative double flash method that takes advantage of both the lower sensitivity and faster recovery kinetics of cones[19,29,30]. In this way it is easier to isolate truly dark-adapted cone-mediated responses. We noticed that in Ames' or Locke's solutions without any blockers, the details of the recovery waveform were somewhat affected during the course of the experiment by the use of bright probe and test flashes. This complicated the subtraction analysis to isolate the cone responses. However, removing the glial component by barium helped to stabilize the tail of the responses indicating that the variability was due to the Müller cell component. In this way it was possible to obtain dark-adapted cone-driven responses in WT mice (**Figure 3**). Cone responses isolated by double flash technique from WT mice appeared smaller as compared to those recorded from WT mice by using background light to suppress rod activity[17,37]. This difference can be explained by a well-characterized effect of background light that slowly (within 10 min) enhances cone response amplitudes probably due to removal of the rod-mediated suppression of cone responses[37-39].

In summary, the method demonstrated here makes possible *ex vivo* electrophysiological recordings to study the function of the retina. In the future, we hope that many more laboratories will adapt this powerful method to study the physiology and pathology of animal and human retina and to advance our understanding of retinal function and develop better therapies for blinding diseases.

Disclosures

Washington University in St. Louis has a license agreement with Xenotec, Inc. and may receive a royalty from the sale of the *ex vivo* adapter.

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References

1. Armington, C. L. *The Electroretinogram by John C. Armington.*. Elsevier Science & Technology Books, (1974).
2. Einthoven, W., Jolly, W.A. The form and magnitude of the electrical response of the eye to stimulation by light at various intensities. *Q J Exp Physiol.* **1**, 43 (1908).
3. Granit, R. The components of the retinal action potential in mammals and their relation to the discharge in the optic nerve. *J. Physiol.* **77**, 207-239 (1933).
4. Penn, R. D., & Hagins, W. A. Signal transmission along retinal rods and the origin of the electroretinographic a-wave. *Nature.* **223**, 201-204 (1969).
5. Stockton, R. A., & Slaughter, M. M. B-wave of the electroretinogram. A reflection of ON bipolar cell activity. *J. Gen. Physiol.* **93**, 101-122 (1989).
6. Robson, J. G., & Frishman, L. J. Response linearity and kinetics of the cat retina: the bipolar cell component of the dark-adapted electroretinogram. *Vis. Neurosci.* **12**, 837-850 (1995).
7. Green, D. G., & Kapousta-Bruneau, N. V. A dissection of the electroretinogram from the isolated rat retina with microelectrodes and drugs. *Vis. Neurosci.* **16**, 727-741 (1999).
8. Steinberg, R. H., Schmidt, R., & Brown, K. T. Intracellular responses to light from cat pigment epithelium: origin of the electroretinogram c-wave. *Nature.* **227**, 728-730 (1970).
9. Wilson, W. S., Shahidullah, M., & Millar, C. The bovine arterially-perfused eye: an *in vitro* method for the study of drug mechanisms on IOP, aqueous humour formation and uveal vasculature. *Curr. Eye Res.* **12**, 609-620 (1993).

10. Frank, R. N., & Dowling, J. E. Rhodopsin photoproducts: effects on electroretinogram sensitivity in isolated perfused rat retina. *Science.* **161**, 487-489 (1968).
11. Donner, K., Hemila, S., & Koskelainen, A. Temperature-dependence of rod photoresponses from the aspartate-treated retina of the frog (Rana temporaria). *Acta Physiol. Scand.* **134**, 535-541, doi: 10.1111/j.1748-1716.1998.tb08528.x (1988).
12. Green, D. G., & Kapousta-Bruneau, N. V. Electrophysiological properties of a new isolated rat retina preparation. *Vision Res.* **39**, 2165-2177 (1999).
13. Hamasaki, D. I. The effect of sodium ion concentration on the electroretinogram of the isolated retina of the frog. *J. Physiol.* **167**, 156-168 (1963).
14. Luke, M. *et al.* The isolated perfused bovine retina--a sensitive tool for pharmacological research on retinal function. *Brain Res. Brain Res. Protoc.* **16**, 27-36, doi: 10.1016/j.brainresprot.2005.09.001 (2005).
15. Bastian, B. L., & Fain, G. L. Light adaptation in toad rods: requirement for an internal messenger which is not calcium. *J. Physiol.* **297**, 493-520 (1979).
16. Arden, G. B. Voltage gradients across the receptor layer of the isolated rat retina. *J. Physiol.* **256**, 333-360 331 (1976).
17. Vinberg, F., Kolesnikov, A. V., & Kefalov, V. J. *Ex vivo* ERG analysis of photoreceptors using an *in vivo* ERG system. *Vision Res.* **101**, 108-117, doi: 10.1016/j.visres.2014.06.003 (2014).
18. Nymark, S., Heikkinen, H., Haldin, C., Donner, K., & Koskelainen, A. Light responses and light adaptation in rat retinal rods at different temperatures. *J. Physiol.* **567**, 923-938, doi: 10.1113/jphysiol.2005.090662 (2005).
19. Heikkinen, H., Nymark, S., & Koskelainen, A. Mouse cone photoresponses obtained with electroretinogram from the isolated retina. *Vision Res.* **48**, 264-272, doi: 10.1016/j.visres.2007.11.005 (2008).
20. Wang, J. S., & Kefalov, V. J. An alternative pathway mediates the mouse and human cone visual cycle. *Curr. Biol.* **19**, 1665-1669, doi: 10.1016/j.cub.2009.07.054 (2009).
21. Bolnick, D. A., Walter, A. E., & Sillman, A. J. Barium suppresses slow PIII in perfused bullfrog retina. *Vision Res.* **19**, 1117-1119 (1979).
22. Newman, E. A. Potassium conductance block by barium in amphibian Muller cells. *Brain Res.* **498**, 308-314 (1989).
23. Oakley, B., 2nd, Katz, B. J., Xu, Z., & Zheng, J. Spatial buffering of extracellular potassium by Muller (glial) cells in the toad retina. *Exp. Eye Res.* **55**, 539-550 (1992).
24. Nymark, S., Haldin, C., Tenhu, H., & Koskelainen, A. A new method for measuring free drug concentration: retinal tissue as a biosensor. *Invest. Ophthalmol. Vis. Sci.* **47**, 2583-2588, doi: 10.1167/iovs.05-1116 (2006).
25. Walter, P., Luke, C., & Sickel, W. Antibiotics and light responses in superfused bovine retina. *Cell. Mol. Neurobiol.* **19**, 87-92 (1999).
26. Luke, M. *et al.* The safety profile of alkylphosphocholines in the model of the isolated perfused vertebrate retina. *Graefes Arch. Clin. Exp. Ophthalmol.* **248**, 511-518, doi: 10.1007/s00417-009-1246-8 (2010).
27. Januschowski, K. *et al.* Electrophysiological toxicity testing of VEGF Trap-Eye in an isolated perfused vertebrate retina organ culture model. *Acta Ophthalmol.* **92**, e305-311, doi: 10.1111/aos.12300 (2014).
28. Kolesnikov, A. V., & Kefalov, V. J. Transretinal ERG recordings from mouse retina: rod and cone photoresponses. *J Vis Exp.*, doi: 10.3791/3424 (2012).
29. Koskelainen, A., Hemila, S., & Donner, K. Spectral sensitivities of short- and long-wavelength sensitive cone mechanisms in the frog retina. *Acta Physiol. Scand.* **152**, 115-124, doi: 10.1111/j.1748-1716.1994.tb09790.x (1994).
30. Lyubarsky, A. L., Falsini, B., Pennesi, M. E., Valentini, P., & Pugh, E. N., Jr. UV- and midwave-sensitive cone-driven retinal responses of the mouse: a possible phenotype for coexpression of cone photopigments. *J. Neurosci.* **19**, 442-455 (1999).
31. Lyubarsky, A. L., Daniele, L. L., & Pugh, E. N., Jr. From candelas to photoisomerizations in the mouse eye by rhodopsin bleaching *in situ* and the light-rearing dependence of the major components of the mouse ERG. *Vision Res.* **44**, 3235-3251, doi: 10.1016/j.visres.2004.09.019 (2004).
32. Azevedo, A. W., & Rieke, F. Experimental protocols alter phototransduction: the implications for retinal processing at visual threshold. *J. Neurosci.* **31**, 3670-3682, doi: 10.1523/JNEUROSCI.4750-10.2011 (2011).
33. Carter-Dawson, L. D., & LaVail, M. M. Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. *J. Comp. Neurol.* **188**, 245-262, doi: 10.1002/cne.901880204 (1979).
34. Fain, G. L., Matthews, H. R., Cornwall, M. C., & Koutalos, Y. Adaptation in vertebrate photoreceptors. *Physiol. Rev.* **81**, 117-151 (2001).
35. Calvert, P. D., Strissel, K. J., Schiesser, W. E., Pugh, E. N., Jr., & Arshavsky, V. Y. Light-driven translocation of signaling proteins in vertebrate photoreceptors. *Trends Cell Biol.* **16**, 560-568, doi: 10.1016/j.tcb.2006.09.001 (2006).
36. Schneeweis, D. M., & Schnapf, J. L. The photovoltage of macaque cone photoreceptors: adaptation, noise, and kinetics. *J. Neurosci.* **19**, 1203-1216 (1999).
37. Heikkinen, H., Vinberg, F., Nymark, S., & Koskelainen, A. Mesopic background lights enhance dark-adapted cone ERG flash responses in the intact mouse retina: a possible role for gap junctional decoupling. *J. Neurophysiol.* **105**, 2309-2318, doi: 10.1152/jn.00536.2010 (2011).
38. Gouras, P., & MacKay, C. J. Growth in amplitude of the human cone electroretinogram with light adaptation. *Invest. Ophthalmol. Vis. Sci.* **30**, 625-630 (1989).
39. Peachey, N. S., Goto, Y., al-Ubaidi, M. R., & Naash, M. I. Properties of the mouse cone-mediated electroretinogram during light adaptation. *Neurosci. Lett.* **162**, 9-11 (1993).

