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Simultaneous Ex Vivo Functional Testing of Two Retinas by In Vivo Electroretinogram System

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Abstract:	In vivo electroretinogram (ERG) signal is composed of several overlapping components originating from different retinal cell types, as well as noise from extraretinal 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.
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TITLE:

Simultaneous *Ex Vivo* Functional Testing of Two Retinas by *In Vivo* Electroretinogram System

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SHORT ABSTRACT:
Ex vivo ERG can be used to record electrical activity of retinal cells directly from isolated intact retinas of animals or humans. We demonstrate here how common *in vivo* ERG systems can be adapted for *ex vivo* ERG recordings in order to dissect the electrical activity of retinal cells.

LONG 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.

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

1.1) Prepare solution for retina perfusion fresh on the day of the experiment. Use distilled and deionized water. Use one of the following three solutions.

1.1.1) Prepare Bicarbonate-containing Ames' solution (1 L): 1 bottle of Ames' media and 1.9 g of $NaHCO_3$,

1.1.2) Prepare Locke's solution (in mM): 112.5 NaCl, 3.6 KCl, 2.4 $MgCl_2$, 1.2 $CaCl_2$, HEPES, 20 mM $NaHCO_3$, 3 Na succinate, 0.5 Na glutamate, 0.02 EDTA, and 10 glucose, 0.1% MEM vitamins and amino acids

1.1.3) Prepare HEPES-buffered Ringer solution: 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.

1.2) Prepare solution for electrodes[28] (in mM): 140 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 room temperature for several months.

1.3) Prepare and test the specimen holder.

1.3.1) 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.

1.3.2) Wait until the glue is almost dry (about 4 minutes) 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.

1.3.3) 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.

1.3.4) Measure the resistance and voltage between the leads of each electrode pair using a multimeter (Figure 1B). Resistance should be below 100 kΩ and the voltage below 10 mV if the channels are bubble-free and the electrodes are in good conditions.

1.4) 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).

1.5) 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 ^{o}C and place it on the heating plate or on the top of the light stimulation unit in water bath set to 37 – 39 ^{o}C[17].

1.5.1) Prime the perfusion lines by filling them with perfusion media to initiate the gravity-driven flow.

1.5.1.1) 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.

1.5.1.2) 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.

1.6) 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

2.1) 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.

2.2) 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.

2.3) For a typical experiment, keep animals in 12/12 hours dark/light cycle and dark-adapt them for 6–12 hours 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).

2.3.1) 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).

2.4) 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.

2.5) 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.

2.5.1) 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 minutes in the solution without significant effects on the response properties.

2.6) 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.

2.7) 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.

2.8) 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

3.1) 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.

3.2) 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 minutes before starting the data collection so that retinal temperature and function reach a stable state.

3.2.1) 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 1000 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).

3.2.2) 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].

3.3) As experiments last typically from 30 minutes 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

4.1) 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.

4.2) 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 (Figures 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 ms 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.

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($Cdsm^{-2}$) 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 $Cdsm^{-2}$ to photons μm^{-2}.

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.

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 designed recently 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 minutes) 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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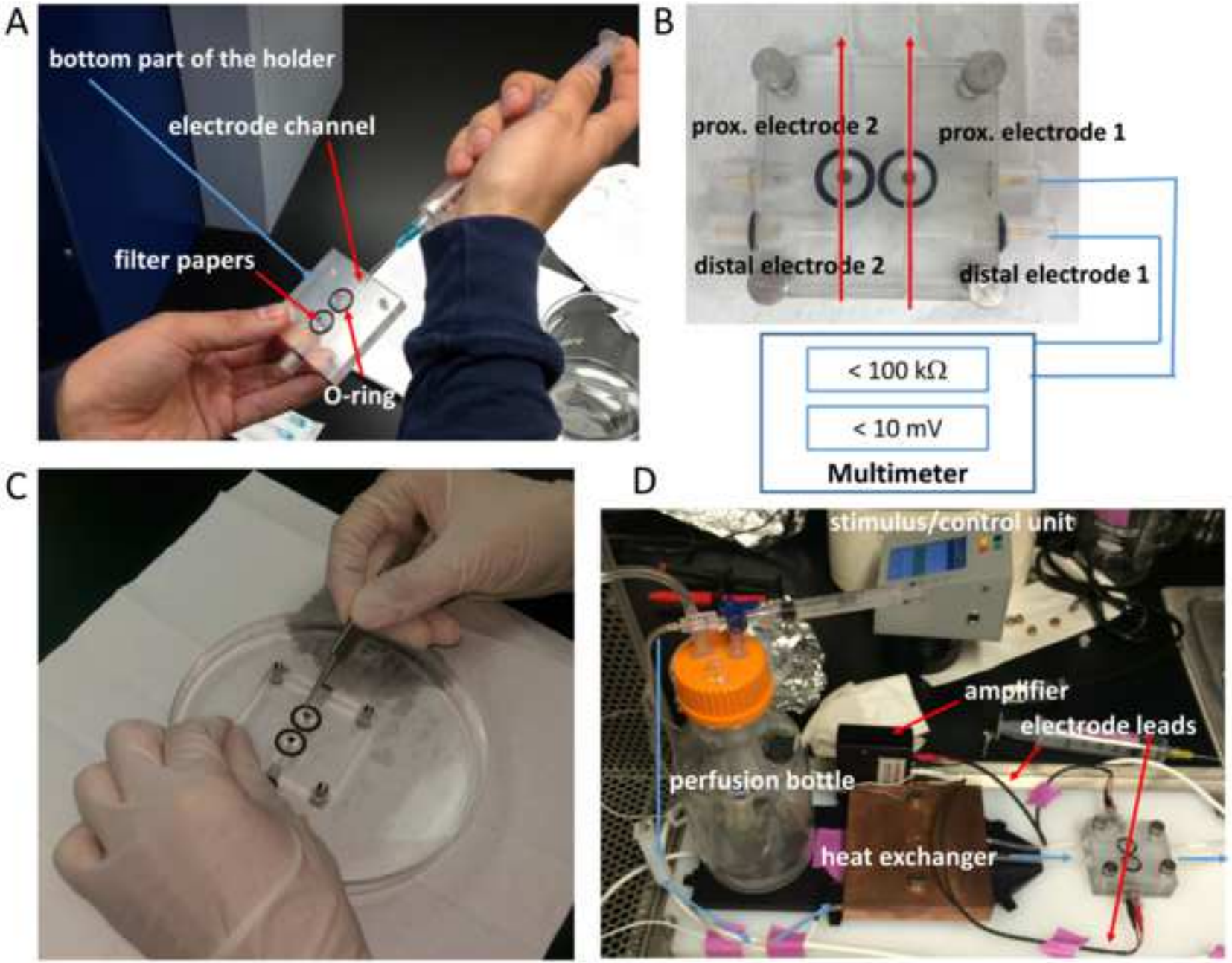

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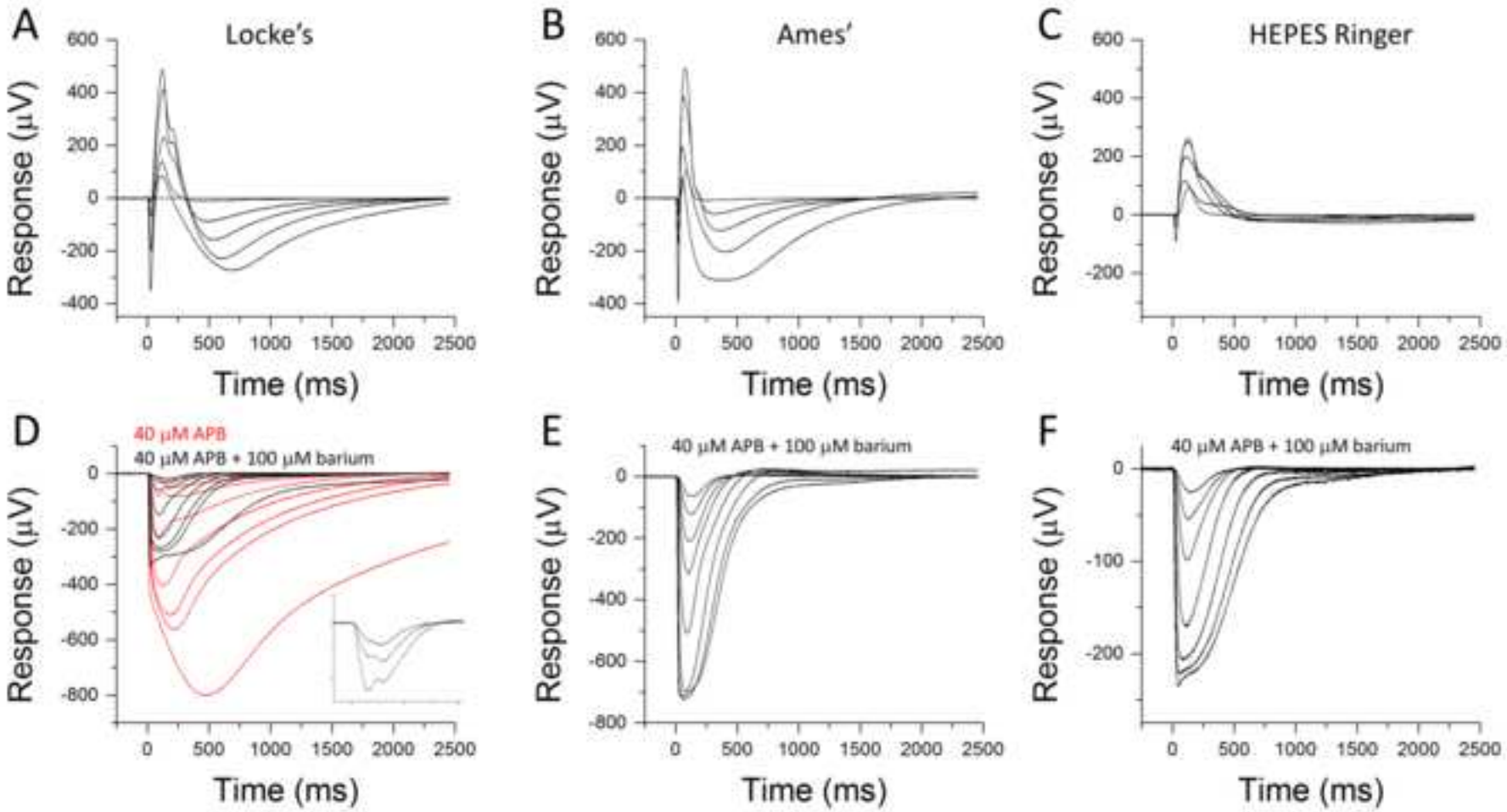

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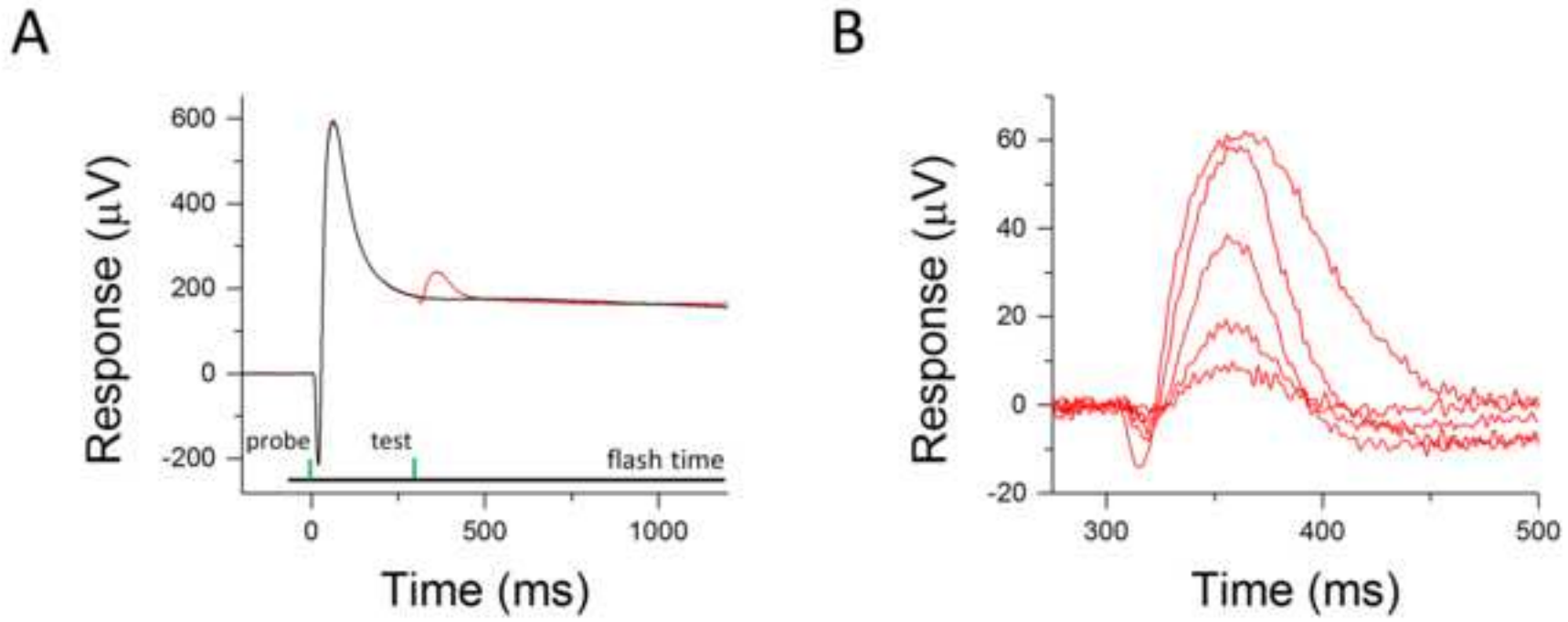

Excel Spreadsheet- Table of Materials/Equipment

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In vivo ERG system	OcuScience	HMsERG	www.ocuscience.us/id77.html
In vivo ERG system	LKC Technologies	UTAS-E 3000	www.lkc.com/products/UTAS/bigshot.html
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Prowler Night Vision Scopes	B.E. Meyers Electro Optics	D4300-I	Military grade product.
Red filter	Rosco Laboratories	Roscolux #27 Medium Red	May be used instead of IR system
Red head light	OcuScience	ERGX011	www.ocuscience.us/catalog/i29.html
Microscissors	WPI, Inc.	500086	www.wpiinc.com/
Dumont tweezers #5	WPI, Inc.	14101	
Razor blades	Electron Microscopy Sciences	72000	www.emsdiasum.com
Scale	Metler Toledo	AB54-S/FACT	May use any brand
pH meter and electrode	Beckman Coulter	pHI 350	May use any brand
NaCl	Sigma-Aldrich	S7653	May use any brand
KCl	Sigma-Aldrich	60129	May use any brand
$MgCl_2$	Sigma-Aldrich	63020	1.0 M solution
CaCl2	Sigma-Aldrich	21114	1.0 M solution
EDTA	Sigma-Aldrich	431788	May use any brand
HEPES	Sigma-Aldrich	H3375	May use any brand
Sodium Bicarbonate	Sigma-Aldrich	S6297	May use any brand
Ames medium	Sigma-Aldrich	A1420	May use any brand
$BaCl_2$	Sigma-Aldrich	B0750	May use any brand
DL-AP4	Tocris Bioscience	101	May use any brand
Succinic acid disodium salt	Sigma-Aldrich	224731	May use any brand

L-Glutamic acid	Sigma-Aldrich	G2834	May use any brand
D-(+)-Glucose	Sigma-Aldrich	G7528	May use any brand
Leibovitz culture medium L-15	Sigma-Aldrich	L4386	May use any brand
MEM vitamins	Sigma-Aldrich	M6895	
MEM amino acids	Sigma-Aldrich	M5550	
Carbogen	Airgas	UN3156	5% CO_2

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Author(s): Frans Vinberg, Vladimir Kefalov

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We thank the editor and all the reviewers for their comments. We have revised the manuscript based on all comments (see accompanying manuscript file where all the changes have been tracked) and provide a detailed answer to comments and concerns raised by editor and reviewers below.

Editorial comments:

1. More detail is needed in a number of steps:

We have now given more details in a number of steps related to the retina dissection and stimulation taking into account the very helpful comments by reviewers 2 and 3.

a) 2.3: How are mice euthanized? How do you dissect the eyes with tweezers?

This information is now provided.

b) 3.1: How is the software configured? (ie, what are you clicking to configure the software in this example?)

We have now given clearly the parameter values related to data acquisition as well as stimulation parameters in units typically used in a commercial *in vivo* system. However, we feel that giving details of how to configure a specific software will not be helpful as different *in vivo* systems have different software interfaces.

c) 3.2: Please give an example of stimulus parameters to use.

This information is now provided.

d) 3.3: How long should the experiment last? Are you recording the signals? ("Follow" is vague.)

We have changed "follow" to "monitor" and give a rough time scale for typical experiments.

e) Line 204: Typo—should be Figure 2 not Figure 7

Thank you, this is now corrected.

2) Figure 2 legend says the red lines in 2D are the samples in APB w/o barium, but the labels in the figure say the opposite. Either the legend or the figure labeling is incorrect.

We apologize for the mix up in the color scheme in Figure 2D, the colors are now corrected in the Figure.

3) Formatting:
a) In vivo and ex vivo should be in italics throughout.

We changed the formatting as requested.

b) First 2 lines of protocol (above the ethics statement) should be a note in section 2.

The information about the housing and dark adaptation of the animals is now part of section 2.3. The mouse strain information is now in the Representative Results section.

c)1.4 & 1.5 plus part of 1.3 should be substeps 1.3.1, 1.3.2 & 1.3.3 under 1.3) Prepare and test the specimen holder.

We have revised the numbering according to the reviewer's suggestion.

d) 2.4: Split into two steps

We have now split 2.4 into two steps.

4) Discussion: Which protocol steps are most critical?

We describe now the most critical steps in the first paragraph of Discussion.

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Reviewers' comments:

Reviewer #1:

We thank reviewer #1 for the supportive comments and have addressed all minor concerns (see below).

Manuscript Summary:

This is a timely and important technique that addresses the major limitation of conventional in vivo ERG, its reproducibility.

This approach not only improves the stability of the retinal field potential, but also enables the testing of different pharmacological agents.

Major Concerns:

none

Minor Concerns:

I am not sure if the authors mention one of the additional components of the ERG, the oscillatory potentials (OPs). These are the oscillating deflections riding of top of the b-wave and reflect the activity of the neurons in the inner retina, presumably inhibitory amacrine cells. Does this technique also reveal this important component of the ERG? From provided illustrations, it's hard to discern their presence. Please, comment.

This is a very good comment and we have been puzzled why OPs are not so prominent under *ex vivo* conditions. In contrast, the b-wave driven by ON bipolar cells appears very strong and stable indicating that inner retina signaling can be preserved *ex vivo*. Though beyond the scope of this paper, developing experimental protocols that preserve OPs and enable their electrophysiological characterization *ex vivo* will be a very useful addition to the range of studies possible with this technique. We have now discussed the lack of OPs in our *ex vivo* recordings in the Discussion.

Additional Comments to Authors:
Great work and highly appealing to many researchers studying retinal function.

Reviewer #2:

We thank reviewer #2 for the helpful comments and have used them to improve the quality of the manuscript by giving more details about the sample preparation and the design of the specimen holder.

Manuscript Summary:
In this article authors describe the use of isolated retinal tissues to record light responses from rod- or cone- or mixed photoreceptor driven retinal circuits. The experimental details regarding the isolation of the retina and mounting of tissues into the chamber are rather scant.

Major Concerns:
Overall it appears as though a great simplification has been given. Is the isolated retina chamber to be purchased? The website given shows $11,000 for this part. No dimension or schematics are given, making this appear as a commercial for the product rather than a technique that can easily be repeated by readers of this article. Authors should include the important and essential characteristics that would allow readers to make their own simple chambers.

See our reply below.

Page 3. Lines 103-106. The description of the specimen holder is extremely sparse and without a schematic and some dimensions, cannot be created given the level of the description. The size and curvature of the domes, the materials and methods for inserting the electrodes all need to be clear. The mechanism by which the retinas are held in place and the electrical isolation of the two sides must also be made clear.

We have now referred to the mechanical drawings published recently by us (Vinberg et al. 2014, Vision Research). In addition, the specimen holder and electrodes are commercially available. We have provided additional details about the sample preparation and retina mounting procedures. The specimen holder has been designed to keep retinas in place without any extra support and following our description here with the available specimen holder it is very unlikely that the retina will displace during the experiment. We have also explained why and where the O-rings are in the specimen holder. It is an important feature of the holder that allows recording of low-noise and large amplitude *ex vivo* ERG signal.

Minor Concerns:
Page 4 lines 138-9. Also unclear and poorly written,

We have revised this section in an effort to clarify it.

Page 4 line 143, sentences as written suggests we immerse tweezers in the media. Are the dissections taking place within the large petri dish in order that the retinas be mounted on domes? If so state that the retina must remain always within the fluid of the perfusion media during dissection and mounting.

We have clarified this part of the manuscript.

Should the media be oxygenated prior to pouring it? (line 138)

We have not oxygenated the media here for the dissection. We make this clear now in the manuscript.

Explain the general stages of preparation…. Why is the petri dish necessary, how long will the chamber be in there?

These steps have now been clarified.

A schematic of the upper and lower retinal holding chamber is necessary. Some sense of scale as well.

See above.

Line 153: Which side of the retina touches the dome? How can you tell which side is which? Is the RPE remaining on the retina? If the RPE supplies the retina with chromophore and lights are used to perform the mounting, how will the visual pigment be regenerated.

The retina mounting procedure has been revised to include more details. RPE will mostly detach from the retina during the dissection process (at least for mouse) and thus the RPE-mediated visual cycle is not functional during the *ex vivo* recordings. We do not find differences between using dim red light or IR light for preparation suggesting that the dim red light used during the mounting etc. does not bleach significant amount of the pigment. 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 RPE-driven regeneration is not an issue. In addition, as we have shown previously, cone pigment regeneration can still take place in the isolated retina via the Muller cell

visual cycle. This is now mentioned in section 3.2.

There appears to be black o-rings, what size are these and what, and how do they accomplish their purpose?

See above.

Page 5 Line 198 The Ames vs. Lockes buffer results are in contrast to published works on single rod photoreceptors. Please reconcile the difference of these two approaches. Either one is less sensitive, or the retinas/cells have been damaged. The recovery times are substantially faster in Ames.

This is already discussed in Azevedo et al. paper (J Neuroscience 2011 Mar 9;31(10):3670-82) where both single cell and transretinal recordings were used. We have mentioned this work in the Discussion but feel that further discussion would be beyond the scope of this manuscript.

Fig 3. The typical isolated cone driven ERG is about 35% of the dark adapted ERG (b-wve), yet in your Fig 3 b, it is only 10%. Can you explain this very small cone response?

This is now explained in Discussion.

If you block potassium flux from Muller cells with barium, then isn't there an elevated potassium level in the retina that will alter the light response?

All pharmacology has potentially some side-effects. There are always pros and cons related to any technique. However, several publications indicate that transretinal ERG with barium provides comparable results to single cell recordings. One possible explanation is that the excess potassium is being removed from the isolated retina by the perfusion solution, i.e. the extracellular potassium concentration is set by the perfusion media.

Reviewer #3:

We thank reviewer #3 for the extensive specific comments. We have revised the manuscript accordingly and provide explanation for the more significant changes below in reply to the reviewer's specific comments. We now provide substantially more details about the light stimulation that should help to choose correct stimulation in *ex vivo* recordings performed with different commercial in vivo ERG systems. We decided to remove the conclusions about fractional sensitivity and kinetics due to reasons explained below.

In this manuscript the investigators describe how commercially available and widely used in vivo electroretinography setups could be with moderate additions/changes used for simultaneous ex vivo transretinal recording from two isolated retinas. Ex vivo ERG allows pharmacological isolation of components originating from certain cell types as well as testing of the efficacy and safety of potential therapeutic drugs. There is rapidly growing interest towards both of these, and therefore the description of the ex vivo ERG protocol may be of great interest and useful for a wide audience and especially to those already having an in vivo ERG setup. The protocols and procedures are explained clearly in the text (except light stimulation, see below), and it is my understanding that the reader should be able to repeat the experiments in her/his own setup based on these descriptions.

Specific comments:
Lines 113-4. It might be worth emphasizing here that even small bubbles in the electrode space can increase noise and therefore should be avoided (though this comes less explicitly on lines 180-1).

This issue is now mentioned in section 1.3.3 and in the Note at the end of section 3.

Lines 124-8. It might be useful to say that in order to keep the perfusion rate relatively constant the perfusate bottle should be placed high enough above the specimen chamber level, e.g. ten times higher compared to the change of solution level in the bottle during the experiment (Fig. 1D may give a wrong impression on the appropriate location of the bottle).

This is a very good point that we also considered during the development of the adapter. We now discuss explicitly how significant gravitational drive is achieved with specific *in vivo* systems in section 1.5.

Line 148. I find the sentence "Cut a small slit between the retina and sclera" confusing. What is meant with that?

In an effort to clarify, this sentence was replaced by: 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.

Lines 146-151. Retinal pigment epithelium is not mentioned at all. How is the retina isolated from the pigment epithelium?

Yes, the retina detaches from the pigment epithelium during dissection. This is now mentioned in section 2.5. Dissection details. As now discussed in section 3.2, this does not affect the light responses in a typical experiment as the amount of pigment bleached by the test flashes is negligible.

Lines 141-166. No attention is paid on the time spent in the isolation procedure. Is it critical that isolation is completed very rapidly? How does it affect the state of the retinal cells if the isolation is done "slowly"?

This is an important point that has been now addressed in section 2.5.

Line 170. The acquisition frequency band is not given right. I assume that it was intended to tell that the acquisition frequency range is from 0 to 1-10 kHz (taking into account that on line 178 the reader is given advice to follow the baseline drift).

The confusing phrasing in the original manuscript has now been corrected. We meant that any sampling frequency between 1 and 10 kHz can be chosen.

Lines 169-182. Practically nothing is said about light stimulation. In ERG all the photoreceptor cells in the active recording area should be stimulated equally. The authors should give some general description how this can be achieved in the commercial in vivo setups and how the level of photoreceptor stimulation should be estimated. How do the possible reflections in the recording chamber affect the homogeneity of stimulus light at the retinas and how can the reader achieve appropriate light stimuli at each of the two retinas?

The original idea was to give stimulus in units that are independent of stimulation light (and we should have mentioned that we used green 530 nm light) but we agree that in this type of article readers should be given stimulation parameters that can be directly used in their *in vivo* system's software. We have now clarified how to choose stimulation in comparison to *in vivo* recordings and give approximate range of values in photopic luminous energy (Cd s/m^2). We also refer to our recent paper which includes extensive explanation of how photopic luminous energy can be converted to pigment isomerizations which is ultimately the brightness that photoreceptors detect. Recording from two retinas simultaneously is of course only possible with *in vivo* systems that can collect data simultaneously from both eyes of the animal (e.g. LKC and Ocuscience). The location of the retinas in the *ex vivo* experiments is very close to the location of the eyes of a live animal in the Ganzfeld sphere during *in vivo* recordings. Therefore, the incident light uniformity and energy are comparable between the two methods. The reflections in the specimen holder could indeed affect the pigment isomerizations as discussed in Vinberg et al., 2014. To address this issue, the commercial adapter

includes a black cover with apertures above the retinas which reduce light scattering and facilitate accurate calibration of light strength.

Line 206-8. The sentence "However, fractional sensitivity, kinetics and stability were comparable across all perfusion conditions used here" is confusing. It sounds to me that this sentence refers to the data of Fig. 2D-E, i.e. in the presence of both APB and barium, not to "all perfusion conditions". Further, the terms "fractional sensitivity, kinetics and stability" lack attributes they are referring to. The authors should formulate the sentence more precisely to make it understandable also to readers not familiar with photoreceptor physiology.

We agree that we were not clear enough with the definition of fractional sensitivity and kinetics. In addition we didn't perform a comprehensive set of experiments that would allow us to draw conclusions about sensitivity that somewhat varies between the experiments. We think that there actually might be about 2-fold higher fractional sensitivity in Ames as compared to Locke's and Ringer (although kinetics as judged from time-to-peak of a dim flash response appears invariant across all perfusion solutions). However, we feel that the subtle differences in response properties under different media are outside the scope of this paper. To simplify the text, we have removed the related conclusions about fractional sensitivity and kinetics.

Lines 226-236. The values given for flash strengths are not useful without telling the stimulus light composition. Please, give that for both the data in Fig. 2 and Fig. 3.

See above.

Lines 227-231. The colours in the text do not correspond to those in Fig 2D. Switch the colours of the curves in the figure (black and red).

This is now corrected.

Lines 233-237. It is told in the text that the recordings shown in Fig. 3 are cone responses. To me it seems that the recordings show timely overlapping components from cones and bipolar cells, i.e. APB was not present in the perfusate. I would like to suggest the authors to add double flash cone data from recordings with APB present in order to show also "pure" cone responses.

We indeed isolated here cone-mediated responses in the absence of APB because there is already a comprehensive study by Heikkinen et al. 2008 about how to isolate pure cone responses in mouse transretinal recordings. We cite that work but stick with the original response family containing the b-waves. We have made the text more clear about what were recorded.

Line 241. The first sentence in Discussion gives me the impression that the two retinas recorded from were perfused with different solutions. I cannot say whether it was the case in reality, but it might be worth emphasizing this possibility in the text.

In this study we did not perfuse two retinas from the same mouse with different solutions. This is now explicitly stated in the Discussion but as suggested by the reviewer we now mention this possibility.

Line 265. See the comment concerning lines 206-8.
The discussion about fractional sensitivity is now removed (see above).

Minor:
On line 7 in the Abstract, I find the words "different standard" needless.
On the last line of the Abstract, I would replace "different" with "certain".
Line 40. I find the words "different standard" needless.
Line 248. I would replace "through" with "above".
Line 263. I would replace "meant" with "intended".
here is that any effect of channel modulation in mouse rods by calcium is quite small

We have done these revisions.

Reviewer #4:

We thank reviewer 4 for the comments and have revised the manuscript as suggested. It is true that the original manuscript did not clearly state whether electrodes are included in the commercial system and that was because all the commercial references were removed in the editorial process from the originally-submitted text.

Manuscript Summary:

The authors describe a nice and easy method for recording light responses with high signal-to-noise ratio from isolated retinas using commercially available equipment and software. The usefulness of such "ex vivo" ERG recordings from intact retinas has increased enormously since they were first introduced in the late 1960's thanks to the analytical understanding of the components, enabled by insights from more invasive (and more demanding) techniques. It now provides an attractive possibility, e.g. for fast testing of transgenic mouse strains, or for successful study of human retinas, with respect to the functioning of rods and cones as well as other retinal elements. Parallel recording from two retinas as introduced by the authors minimizes biological variation in treatment/control comparisons and reduces the number of experimental animals used.

Major Concerns:

N/A

Minor Concerns:

I have a few minor comments:

1. Abstract last sentence: It is not the method here described that is used to remove specific ERG components, but pharmacological treatments.

The last line of the Abstract was revised to address this concern.

2. Introduction: I think it would be worthwhile to mention how the ERG signal arises as a field potential mainly reflecting light-induced changes in radial extracellular current components in the resistive retinal tissue.

This is now mentioned in the Introduction.

3. Lines 52-53: "the ERG signal" should be "the ERG response to the onset of light"
4. Line 65: "modalities" is not a good expression: maybe rather "recording configurations".
5. Line 66: "Transretinal ERG from the intact, isolated retina (ex vivo ERG)…"
6. Line 67: "Few studies" should be "A few studies"

All of these (3-6) changes have been now made.

7. Lines 80-81: although the "power" of the b-wave is provided by Müller cells, it sounds somewhat misleading to refer to this (and the slow PIII) as questions of Müller cell function.

The text describing the use of *ex vivo* ERG to assess Muller cell function has been clarified. We have now described the involvement of Muller cells in generation of the negative slow PIII component that is prominent in the *ex vivo* ERG signal (see Figure 2D). In this way it is explicit what we mean by suggesting that *ex vivo* ERG can be used to study Muller cell function in the intact retina.

8. 8. Line 246: "custom-build" should be "custom-built".
9. Line 263: "the" is missing in "meant to isolate the photoreceptor component…"
10. Line 288: "barium" should not be capitalized
Throughout: Since the names of the originators of the solutions used are Ames, Locke and Ringer, the proper spelling of the respective solutions is Ames' solution, Locke's solution and Ringer's solution (or plainly Ringer).

All of these changes (8-10) have been now made.

The procedures appear to be described in sufficient detail, although this is difficult to judge by just reading. E.g., presumably the electrodes used in the ex vivo adapter are included in the commercial system and need not be separately mentioned?

The electrodes are indeed included in the system and significant amount of effort has been used to design electrodes that are easy-to-use and hard to break in order to make commercialization of this specimen holder possible.

Additional Comments to Authors:
Additional references suggested:
The prototype of the specimen holder was described in:
Bastian, B.L. and Fain, G.L. (1979). Light adaptation in toad rods: requirement for an internal messenger which is not calcium. Journal of Physiology 297: 493-520.

The double-flash technique for isolating cone ERG responses (now attributed to Heikkinen et al., 2008) was developed in:
Koskelainen, A., Hemilä, S. & Donner, K. (1994). Spectral sensitivities of short- and long-wavelength sensitive cone mechanisms in the frog retina. Acta Physiologica Scandinavica 152, 115-124.

Thank you for reminding us of these important references, they have been now added to the manuscript.