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Direct-coupled electroretinogram (DC-ERG) for recording the light-evoked electrical responses of the mouse retinal pigment epithelium

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Cover Letter

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
National Eye Institute
Bethesda, Maryland 20892

03-23-2020

Dear Dr. Aaron Berard,

Thank you for your invitation to submit our protocol entitled “Direct coupled electroretinogram (DC-ERG) for recording the light evoked electrical responses of the mouse retinal pigment epithelium” for consideration for publication in JOVE.

In contrast to the more common electroretinogram recordings the DC-ERG measures retinal pigment epithelium function, which plays a critical role in the pathology of many forms of retinal degeneration. The retinal pigment epithelium (RPE) is a specialized monolayer of cells strategically located between the retina and the choriocapillaris that maintain the overall health and structural integrity of the photoreceptors.

The technique we describe is a variation based on Wu et. al 2004 (“Light-Evoked Responses of the Mouse Retinal Pigment Epithelium). We have added an additional step in preparing the recording electrodes that has greatly improved the stability (reducing noise and drift) and the reproducibility of the recordings. In addition, we provide downloadable files that include our light stimulation protocols for a ganzfeld stimulator (Diagnosys, LLC) and also MATLAB scripts for rapid analysis of the DC-ERG recordings. To demonstrate the technique and the analysis workflow we include previously unpublished data showing representative results in a mouse model of retinoschisis (RS1 KO).

We hope this protocol earns your favorable consideration. Please let me know if there are any questions or concerns.

Yours sincerely,

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TITLE:

Direct-Coupled Electroretinogram (DC-ERG) for Recording the Light-Evoked Electrical Responses of the Mouse Retinal Pigment Epithelium

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KEYWORDS:

DC-ERG, retinal pigment epithelium, electroretinogram, mouse model, c-wave, fast oscillation, light peak, off response

SUMMARY:

Here, we present a method for recording light-evoked electrical responses of the retinal pigment epithelium (RPE) in mice using a technique known as DC-ERGs first described by Marmorstein, Peachey, and colleagues in the early 2000s.

ABSTRACT:

The retinal pigment epithelium (RPE) is a specialized monolayer of cells strategically located between the retina and the choriocapillaris that maintain the overall health and structural integrity of the photoreceptors. The RPE is polarized, exhibiting apically and basally located receptors or channels, and performs vectoral transport of water, ions, metabolites, and secretes several cytokines.

In vivo noninvasive measurements of RPE function can be made using direct-coupled ERGs (DC-ERGs). The methodology behind the DC-ERG was pioneered by Marmorstein, Peachey, and colleagues using a custom-built stimulation recording system and later demonstrated using a

commercially available system. The DC-ERG technique uses glass capillaries filled with Hank's buffered salt solution (HBSS) to measure the slower electrical responses of the RPE elicited from light-evoked concentration changes in the subretinal space due to photoreceptor activity. The prolonged light stimulus and length of the DC-ERG recording make it vulnerable to drift and noise resulting in a low yield of useable recordings. Here, we present a fast, reliable method for improving the stability of the recordings while reducing noise by using vacuum pressure to reduce/eliminate bubbles that result from outgassing of the HBSS and electrode holder. Additionally, power line artifacts are attenuated using a voltage regulator/power conditioner. We include the necessary light stimulation protocols for a commercially available ERG system as well as scripts for analysis of the DC-ERG components: c-wave, fast oscillation, light peak, and off response. Due to the improved ease of recordings and rapid analysis workflow, this simplified protocol is particularly useful in measuring age-related changes in RPE function, disease progression, and in the assessment of pharmacological intervention.

INTRODUCTION:

The retinal pigment epithelium (RPE) is a monolayer of specialized cells that line the posterior segment of the eye and exert critical functions to maintain retinal homeostasis[1]. The RPE supports photoreceptors by regenerating their photon-capturing visual pigment in a process called the visual cycle[2], by participating in the diurnal phagocytosis of shed outer segment tips[3], and in the transport of nutrients and metabolic products between photoreceptors and the choriocapillaris[4,5]. Abnormalities in RPE function underlie numerous human retinal diseases, such as age-related macular degeneration[6], Leber's congenital amaurosis[7,8] and Best vitelliform macular dystrophy[9]. As donor eye tissues are often difficult to obtain solely for research purposes, animal models with genetic modifications can provide an alternative way to study the development of retinal diseases[10,11]. Additionally, the emergence and application of CRISPR cas9 technology now permits genomic introductions (knock-in) or deletions (knock-out) in a simple, one-step process surpassing limitations of prior gene targeting technologies[12]. The boom in the availability of new mouse models[13] necessitates a more efficient recording protocol to non-invasively evaluate RPE function.

Measurement of the light-evoked electrical responses of the RPE can be achieved using a direct-coupled electroretinogram (DC-ERG) technique. When used in combination with conventional ERG recordings that measure the photoreceptor (a-wave) and bipolar (b-wave) cell responses[14], the DC-ERG can define how the response properties of the RPE change with retinal degeneration[15-17] or whether RPE dysfunction precedes photoreceptor loss. This protocol describes a method adapted from the work of Marmorstein, Peachey, and colleagues who first developed the DC-ERG technique[16,18-20] and improves upon the reproducibility and ease of use.

The DC-ERG recording is difficult to perform because of the long acquisition time (9 min) during which any interruption or introduction of noise can complicate the interpretation of the data. The advantage of this new method is that the baselines reach steady state within a shorter amount of time reducing the likelihood that the animal will awaken prematurely from anesthesia and is less prone to bubble formation in the capillary electrodes.

PROTOCOL:

This protocol follows the animal care guidelines outlined in the animal study protocol approved by the Animal Care and Use Committee of the National Eye Institute.

1. Importing light stimulation protocols for DC-ERG

NOTE: Follow the directions below to import the light stimulation protocols for the DC-ERG into the ERG system software (**Table of Materials**). The protocol consists of a 0.5 min pre-stimulus interval, followed by a step of light (10 cd/m^2) for 7 min, and ending with a 1.5 min post-stimulus interval. The light intensity of 10 cd/m^2 (1 $\log_{10}$ cd/m^2) was selected since it evokes approximately half the maximal response for all the components of the DC-ERG in WT mice[18,21]. The c-wave and fast oscillation are of particular interest as the origins of these electrical responses are well characterized and can be isolated and studied further in vitro RPE models (e.g., iPSC-RPE). The application of other light intensities can extract additional information, for instance, the off response undergoes a reversal of polarity at brighter light stimuli and may show differences at the intensity at which this reversal takes place. The user is free to change the light intensity settings at their discretion.

1.1. Open the ERG system software.

1.2. Click on **Database Center**.

1.3. Click on **New** (provide a new database file name). Click on **Save**. The popup box will display: "Database created, do you want to connect to the new database file." Click on **Yes**. The current database name should now reflect the new file name.

1.4. Click on **Transfer In** in the Database Control Center Window.

1.5. Select **Supplementary File 1: LightProtocols - TRANSFER.EXP**. Click on **Open**.

1.6 Click on **Close** (Database Control Center) upon completion of the progress bar.

1.7 Click on the green **Start** button.

1.8 Click on **New** on the **Select Patient Window**. Create a new patient family name describing the mouse model, enter the date of birth (DOB, mm/dd/yyyy), toggle the gender (M/F) button to the appropriate description. Click on **Close** to save the experimental details.

1.9 Click on **Protocols**. The dark-adapted ERG and DC-ERG protocols should now be visible.

1.10 Lastly, place the Long Flash.col file into the following folder C:\ERG User Files\Long Flash.col.

2. Capillary electrode preparation

2.1 Cut the 1.5 mm glass capillaries in half by using a ceramic tile (**Table of Materials**) to score the glass and break them cleanly using a table to provide physical counterforce and to stabilize the glass.

NOTE: Blunt the cut ends as necessary.

2.2 Using a Bunsen burner (**Table of Materials**) allow the heat to make a small bend in the capillary while holding it over the flame with forceps.

3. Filling capillary electrodes

3.1 Connect the vacuum desiccator to the laboratory's vacuum line through an in-line filter (**Table of Materials**).

3.2 Pour 30 mL of Hanks' Balanced Salt Solution (HBSS) **(Table of Materials)** into an open 50 mL conical tube and place it (with the cap removed) into the vacuum chamber.

3.3 Turn on the vacuum and degas the HBSS while turning on the computer and the recording equipment.

3.4 After 5–10 min turn off the vacuum and use the degassed HBSS to fill a 12 mL syringe through an attached 25 G needle. Use it to fill the bases of the electrode holders taking extra precaution not to introduce bubbles.

3.4.1. To do this, remove the threaded screw cap and carefully slide the syringe needle through the silicone rubber gasket to reach the back wall (silver/silver chloride pellet) of the holder.

NOTE: The silver/silver chloride pellets are advantageous over holders that use silver wire as they provide more surface area resulting in a stable low-noise baseline. However, silver/silver chloride pellets require a liquid interface free from air bubbles to achieve a good connection. Therefore, take great care not to introduce air bubbles during this process.

3.4.2. Gradually inject HBSS to fill the entire microelectrode while slowly retracting the syringe needle. Reattach the threaded cap but do not tighten. Reinsert the syringe needle to fill the empty space within the cap with HBSS. Then fill the glass capillary while holding it horizontal to prevent solution from escaping from the other end.

3.4.3. Hold the glass capillary from the bent end and slowly insert the opposite end through the loosened cap and then tighten the screw cap into place.

NOTE: Glass electrodes filled with HBSS maintain lubrication of the mouse's eye and prevent corneal dehydration that would occur with the use of standard gold loop electrodes.

3.5 Position the electrode holders with the capillary electrodes tilted upwards to allow bubbles to flow out. Run the vacuum for 5–10 min to degas. Gas escaping from the glass and plastic surfaces will push HBSS out from the electrode holders.

3.6 Stop and then slowly release the vacuum. Refill the electrode holders and glass capillaries as described previously.

NOTE: Bubbles tend to collect on or near the silicone rubber gasket and can also hide in the grooves of the threaded cap, therefore special attention must be paid to keep these areas bubble-free.

3.7 Install and secure the microelectrode holder into the custom-made T-clip/Magnetic ball joint stand (**Figure 1A**, inset). To make the custom stand for the microelectrode holder, modify a T-clip (5/16”-11/32” OD Tubing) #8 (**Table of Materials**) by removing the black polyacetal clips on one side. Have the cylinder base of the magnetic ball joints machined in half to adjust the height (**Table of Materials**). Secure the modified T-clips to the magnetic ball mounting screws with M3 sized nuts.

3.8. Place the microelectrode holder into the modified T-clip and hold it tightly in place by sliding in approximately a 1-inch tapered wooden handle made from breaking a cotton tipped cleaning stick (**Table of Materials**) at an angle. Use the rare earth magnet cylinder base to securely position the customized electrode holder stand onto the metal plate of the stage enabling 360° rotation on a 180° axis.

4. Test electrodes

4.1 Pour HBSS into a small container (e.g., the cap of the 50 mL conical tube).

4.2 Gently lower the fully assembled, HBSS-filled capillary microelectrodes into the cap containing HBSS to pre-equilibrate the electrodes and place the needle ground electrode (tail/rear leg) and the Ag/AgCl sintered pellet reference electrode (mouth) in the same HBSS to complete the circuit (**Figure 1A**).

NOTE: Perform all subsequent steps under dim red light. Use a red-light flashlight to position the mouse and capillary electrodes. Remember to completely turn off all light sources prior to beginning the recording.

4.3. Select or create an appropriate identifier (family name) to describe the mouse to be tested and select the DC-ERG protocol to be performed by completing the registration according to the following order.

4.3.1. Click on **Protocols**. Select **DC-ERG**. Click on **Run**. A dialog box will pop-up: “Current patient is XXX [DOB: XX/XX/XX) Is this correct for the test being performed?” Click on **Yes**. Then proceed to “Step 1/6.”

4.4. Close the doors to the faraday cage.

4.5. Display impedance mode by clicking on **Impedance** and verify that the values for the mouth reference, tail ground, and recording electrodes are acceptable (see **Figure 1B**).

4.6 Test the baseline stability (noise and drift) by clicking on **Step** (forward arrow) to reach Step 4/6, which is designated "Long Flash No Light."

NOTE: The amount of drift observed when the electrodes are placed in the HBSS bath is generally less than 500 µV per 80 s once they have stabilized and is equivalent to the drift observed when the electrodes are connected to the mouse. Thus, the electrical readout of the electrodes in the HBSS bath are an important indicator of the status of the electrodes. The noise, measured as peak-to-peak, is generally ~ 10–15% greater in the mouse than in the HBSS bath. This is probably due to the addition of motion artifacts from breathing.

4.7 Begin viewing the traces by clicking on **Preview**. The traces should be low noise with a peak-to-peak amplitude <200 µV. A slight drift (<500 µV/80 s) that gradually fades to baseline is acceptable (**Figure 1C**).

5. Mouse and electrode positioning

5.1 Keep the mice overnight in a well-ventilated light-tight box for dark adaptation.

5.2 Anesthetize the animals by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg).

5.3 Apply a drop of 0.5% proparacaine HCl topically to anaesthetize the cornea as well as a drop of 2.5% phenylephrine HCl and 0.5% tropicamide to dilate the pupils.

5.4 Trim the mouse whiskers with scissors to prevent inadvertent twitching from disturbing the glass capillary electrodes during the recording.

NOTE: The DC-ERG stimulus protocol within the ERG system has several built-in stimulus routines that can be selected by clicking on the **Step** (forward arrow) or **Step** (backward arrow). Only Steps 1, 4, and 5 in the software are required to prepare and perform the DC-ERG recording.

5.5 In the ERG system software verify that the correct patient is selected. Click on the green **Protocols** button. Under **Protocol Description** select **DC-ERG.** Then click on **Run**. Verify that this is the correct test being performed by clicking on **Yes**.

5.6 Use Step 1/6 designated Red Light stimulus to turn on the red light inside the dome to help position the mouse and electrodes while observing the changes in impedance.

5.7 Place the mouse on a heated recording table and carefully tent the skin of the rear leg using forceps. Hold the needle electrode firmly in one hand and insert it subcutaneously into the rear leg to secure it into place.

5.8 Place the reference Ag/AgCl electrode (**Table of Materials**) inside the mouth so that the sintered pellet rests along the back cheek and is held in place behind the teeth.

NOTE: Gold wire electrodes should not be used as the mouth reference electrode as they have different impedance characteristics and increase the peak-to-peak noise.

5.9 Prior to placing the capillary electrodes to the mouse's eye, hold the electrode holder with the glass capillaries vertical, flick the electrode holder with the index finger to remove any bubbles that may have been introduced. Fill the tip with HBSS using a 25 G needle attached to a syringe and inspect to ensure that there is no air bubble trapped at the tip. Position the electrode holder stand so that the open tip of the HBSS-filled capillaries are in gentle contact with the cornea.

5.8 Use special precaution to avoid introducing air bubbles by holding the lubricant eye gel dispenser inverted and discard the initial drops. Place a drop of lubricant eye gel on each eye to maintain conductivity and prevent desiccation during the recording.

6. DC-ERG recording

6.1 Click on **Step** (forward arrow) to select Step 5/6 "Long Flash 10 cd 7 min."

6.2 Click on **Impedance**. Use the **Impedance Checking** screen to examine the resistances of the left and right eyes. The impedance values for the recording electrodes at each eye are expected to be similar (~39 kΩ). The impedance values for both the ground and reference electrodes are expected to be less than 10 kΩ).

6.3 Click on **Preview** to view the traces for the left and right eye. Wait for a stable baseline to be achieved (<10 min). Click on **Stop** to exit the trace preview.

NOTE: Abrupt changes in drift direction or aberrant noise in the recording will not improve with time and will require identifying the capillary electrode that requires attention. The most likely cause is a bubble introduced to the tip of the electrode. Refill the tip with HBSS. Click on **Preview** again to check the baseline.

6.4 Click **Run** to start the recording (**Figure 1D**).

7. Data export

7.1 Select the "Patient" (Family Name) describing the mouse recording to be exported.

7.2 Click on **Old Tests**.

7.3 Under **Protocol Description** select **DC-ERG**. Click on the green **Load** button to load the previously acquired data.

7.4 Click on **Step** (forward arrow) to advance to Step 5/6 “Long Flash 10 cd 7 min.”

7.5 Click on **Export**.

7.6 Provide the filename (e.g., filename.csv). A valid filename must begin with a letter, followed by letters, numbers, or underscores. Do not use special characters or hyphens. The data analysis program (DCERG_Analysis.exe) requires that table entries meet the requirements for variable names.

7.7 Place a checkmark next to **Data Table**. Next to separator, select **Tab**. Place checkmarks next to Options (**Titles**, **Vertical**), Include All (**Steps**, **Chans**, **Results**), Data Columns (**Contents**, **Results**, **Sweeps**), and Format (**File**).

7.8 Then click on **Export** (**Figure 1E**). This saves the *.csv file to the C:\Multifocal folder.

8. Data analysis

8.1 Download and install the appropriate runtime installer (**Table of Materials**)

8.2 Download and install the DCERG_Analysis.exe installer.

NOTE: This installs the script that will perform the analysis of the DC-ERG components and creates a shortcut to run the program in the Start Menu folder.

8.3 Click on the shortcut created in the **Start Menu** > **Programs** folder.

8.4 Select the exported data file or files (*.csv) for analysis. Use **Ctrl** + **left** click on the mouse to select more than one file.

NOTE: The executable file generates two types of plots: 1) the raw data is plotted with a best fit line indicating the measured drift; 2) the drift corrected response is plotted after being smoothed with a moving average (with a span of ~5 s). From this plot the amplitudes and time-to-peak of the DC-ERG components are identified: c-wave, fast oscillation, light peak, and off response. The data is then exported in table format to excel where each sheet corresponds to a different mouse recording. These sheets are followed by two summary sheets: (i) compiled DC-ERG amplitudes (mV); (ii) compiled DC-ERG time-to-peaks (light onset, t = 0 min).

REPRESENTATIVE RESULTS:

Figure 2 is a sample dataset from miR-204 ko/ko cre/+ (conditional KO) and wild type (WT) mice. MiR-204 ko/ko cre/+ are mice with a conditional knockout of microRNA 204 in the retinal pigment epithelium. These mice are generated by crossing floxed miR-204 mice (produced by NEIGEF)[22] with VMD2-CRE mice[23]. MiR-204 is highly expressed in the RPE where it regulates the expression of proteins critical for epithelial function that maintain tight junction integrity (e.g., claudins), the maintenance of potassium homeostasis through the expression of Kir 7.1 potassium channels, and the expression of several visual cycle genes (e.g., *LRAT*, *RPE65*)[24].

Since abnormal RPE morphology was reported in several RPE-specific Cre expressing mouse lines[25], we monitored for normal RPE morphology in Cre expressing mice with the WT phenotype. The structural and functional abnormalities of the miR-204 ko/ko cre+ (conditional KO) mouse retina resemble the features found in miR-204 null mice[15] characterized by hyper autofluorescence (lipofuscin-like deposits) and increased microglia localized to the RPE apical surface. In null mice these changes were accompanied with decreased light-evoked electrical responses of the RPE, with minimal alteration to photoreceptor responses (assessed by retinal ERG). Thus, perturbation of miR-204 expression in miR-204 ko/ko cre/+ mice is also expected to alter the electrical response of the RPE.

In the example presented, a mouse is placed on the heated platform and the electrodes are positioned appropriately prior to lowering the dome. Impedance and drift are checked as previously described using the bath solution. Representative "negative" results are shown in **Figure 2A**. In **Figure 2A** (top panel), the trace suffers from minute bubbles in the electrode that increase the peak-to-peak noise in the trace (shaded in blue). In another example (**Figure 2A**, lower panel), when bubbles detach from the surface of the glass and move along the length of the electrode this causes abrupt changes in the direction of the baseline drift that cannot be compensated by drift subtraction. **Figure 2B** shows representative "positive" recordings of WT and miR-204 ko/ko cre/+ mice where the bubbles have been eliminated using the vacuum chamber prior to assembling the microelectrodes into the electrode holder stands.

The best fit line to the initial 25 s (green) is calculated and shown in blue (**Figure 2B**). The drift corrected responses are replotted in **Figure 2C** along with the identification of the amplitudes of the DC-ERG components. Using the DC-ERG technique described in this protocol animals from both WT and miR-204 ko/ko cre/+ strains can quickly be recorded and analyzed.

The c-wave is composed of two components: a hyperpolarization of the RPE apical membrane due to increased potassium conductance in response to a decrease in potassium in the subretinal space due to photoreceptor activity and a separate contribution originating from inner retinal cells (slow P3 component – reflecting the activity of Müller cells). The fast oscillation provides information regarding the hyperpolarization of the RPE basolateral membrane[26], primarily due to changes in the conductance of a Cl transporter called cystic fibrosis transmembrane conductance regulator (CFTR)[27]. The light peak is thought to originate from a change in the concentration of a photoreceptor driven substance[28] that through a second messenger system depolarizes the RPE's basolateral membrane by modulating the activity of Ca^{2+} dependent Cl

channels[21]. Lastly, the off-response is a complex interaction of responses that differ in polarity and vary with light intensity[18].

As expected, reduced expression of K_{ir} 7.1 K^+ channels greatly attenuates the c-wave[29] and fast oscillation as shown in the averaged responses in **Figure 2D**, indicating a significant impairment of the RPE's electrical properties. A summary of the changes in the components of the DC-ERG are provided in **Figure 2E**. The relative amplitudes of the DC-ERG components (normalized to WT) are plotted against the relative two largest light-evoked a-wave amplitudes (1 cd·s/m^2; 10 cd·s/m^2) (normalized to WT) and shown in **Figure 2F–H**. The reduction in the a-wave response to the brightest light intensity (10cd·s/m^2) (**Figure 2F–H, Supplementary Figure S1A,B**) suggests a delay in the recovery of sensitivity due to visual cycle impairment (e.g., resulting from reduced LRAT or RPE65 expression as a result of genomic knockout of miR-204[24,30]).

FIGURE AND TABLE LEGENDS:

Figure 1: The diagram highlights key steps in the DC-ERG protocol. (**A**) Image of the completed circuit accomplished by lowering the recording (glass capillary microelectrodes), reference, and ground electrodes into the same bath solution. This configuration enables preliminary tests to be run (prior to anesthetizing the mouse) to evaluate the characteristic impedance, noise, and drift. Inset (upper left) showing a side-view schematic of the custom microelectrode holder stand. (**B**) Representative image of the Impedance Checking Mode showing the appropriate values for electrode impedances. The impedance in the Left and Right eye electrodes should be comparable, within 3 KΩ of each other (e.g., Left eye: 38.7 KΩ vs. Right eye: 40.36 KΩ). The impedance of the mouth reference electrode should be less than 0.5 KΩ, whereas the tail electrode should be around 2.5 KΩ. (**C**) Representative image of the preview trace (Step 4/6) is shown. Step 4 (Long Flash No Light) is selected as no light is delivered during the preview of this step. The traces should be low noise and may have a slight drift that gradually fades with time to baseline. Once the traces have achieved a constant drift in both channels and become relatively flat, the actual recording can begin. (**D**) Using Step 5/6 (Long Flash 10 cd 7 min) after 0.5 min of darkness, a light step of 10 cd/m^2 is delivered to the mouse for 7 min followed by a return to darkness for 1.5 min. (**E**) Image of the export parameters used to convert the data to a *.csv file. This precise format is required to run the DC-ERG analysis software.

Figure 2: Representative traces and workflow of DC-ERG analysis. Image of a negative DC-ERG result displaying excessive (**A, top panel**) peak-to-peak noise and (**A, bottom panel**) drift. (**B**) Images of positive DC-ERG recording results from a WT and miR-204 ko/ko cre/+ mouse. Generated plots of the raw traces showing the best fit lines (blue) to the initial 25 s (green) prior to light onset. (**C**) Plots of the drift corrected DC-ERG responses for the WT and miR-204 ko/ko cre/+ mice shown in **B**. The amplitudes of the components of the DC-ERG are indicated in the legend. (**D**) Averaged DC-ERG responses of 3–8 months old WT (n = 6) and miR-204 ko/ko cre/+ (n = 6) mice. The DC-ERG components are labeled on the WT trace and the light stimulation parameters are defined below. (**E**) Summary of DC-ERG components taken from recordings of WT and miR-204 ko/ko cre/+ mice. Bar plots represent mean, error bars indicate standard error. The relative amplitudes of the (**F**) c-wave, (**G**) fast oscillation, and (**H**) off response are plotted

against the relative two largest light-evoked a-wave amplitudes (1 cd·s/m^2; 10 cd·s/m^2) (normalized to WT). Significance is indicated by asterisks: (Student's t-test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Supplementary Figure 1: ERG responses of WT and miR-204 ko/ko cre/+ mice. (**A**) Responses of WT (black) and miR-204 ko/ko cre/+ mice (magenta) to 4 ms flashes of light of increasing intensity: 0.0001 cd·s/m^2 (n = 5), 0.001 cd·s/m^2 (n = 5), 0.01 cd·s/m^2 (n = 3), 0.1 cd·s/m^2 (n = 3), 1 cd·s/m^2 (n = 3), 10 cd·s/m^2 (n = 2). (**B**) Averaged a-wave amplitude plotted against flash intensity. (**C**) Averaged b-wave amplitude plotted against flash intensity. (**D**) Averaged time-to-peak of a-wave responses plotted against flash intensity. (**E**) Averaged time-to-peak of b-wave responses plotted against flash intensity. For all plots shown error bars indicate SEM. Significance is indicated by asterisks: (Student's t-test; * = $p < 0.05$).

Supplementary Figure 2: Example of a DC-offset in the power line that can be mitigated with the use of a voltage regulator/power conditioner. (**A**) In the absence of voltage regulation voltage spikes (caused by the use of equipment in an adjacent room e.g., OCT) generate a DC-offset that can interfere with the measurement of the DC-ERG components, especially the light peak. The disruptive offset is magnified on the right. (**B**) With the voltage regulator/power conditioner enabled the initial spike is still noticeable but the damaging DC-offset is removed. The effect of the voltage regulator/power conditioner is magnified and shown to the right.

DISCUSSION:

Critical Steps

A good DC-ERG recording requires stable electrodes that are free from bubbles that create artifacts and unwanted drift as they are extremely sensitive to outgassing and temperature changes. It is essential that a stable baseline is achieved when the electrodes are placed in the HBSS bath solution before proceeding forward with the mouse recording. Small bubbles tend to collect at the base of the capillary electrode or around the silicone gasket and are difficult to see once the electrode holder is fully assembled. When few bubbles are present, lightly flicking the holder will free them for removal. If there are too many bubbles or the drift or noise cannot be removed, it is often better to disassemble the electrode and start over while carefully inspecting for bubbles at each step of the process.

Modifications and Troubleshooting

The following customizations can be made to the setup (**Table of Materials**) in order to improve the fidelity of the DC-ERG recordings. Low-noise cables for microelectrode holders can be used to extend the existing cables from the 32-bit amplifier to the recording table. The additional length enables the careful placement and adjustment of the electrode holder without disturbing their position once the Ganzfeld dome is closed. A voltage regulator/power conditioner can be used to eliminate in line noise and power surges generated from lights or equipment in adjacent rooms being turned on and off (**Figure S2**). Additionally, the tabletop Ganzfeld dome stimulator and the 32-bit amplifier can be placed inside a Faraday cage grounded to the building ground bar to shield against any additional electrical noise.

Limitations of the Method

The DC-ERG can only be recorded faithfully on dark adapted animals meaning that once the light stimulus is turned on there is little that can be done to eliminate undesirable potentials or drift. Another limitation is that the polarity of some of the components of the DC-ERG (light-peak, off-response) is subject to the light intensity used[16]. This means that the greatest deviations from WT may occur at intensities not inherently present at the light intensity that this protocol uses (10 cd/m^2). To this point, the DC-ERG analysis software was designed assuming a negative off response (a response minimum). Brighter light intensities that result in the reversal of polarity of the off response will require the need to alter the included analysis script file.

Significance

The RPE is involved in the homeostatic maintenance of the retinal environment and plays a critical role in the pathology of several retinal diseases. This method explains in detail how to setup a DC-ERG system to record the RPE electrical response that when performed in conjunction with conventional ERG recordings provides an objective measure of outer retinal and RPE function. These measures of RPE functionality can be used to evaluate transgenic mouse lines displaying degenerative phenotypes or to test for drug-efficacy or drug-induced cytotoxicity to the RPE.

ACKNOWLEDGMENTS:

This work was supported by NEI intramural funds. The authors sincerely acknowledge Dr. Sheldon Miller for his scientific guidance, technical advice, and expertise in RPE physiology and disease. The authors thank Megan Kopera and the animal care staff for managing the mouse colonies, and Dr. Tarun Bansal, Raymond Zhou, and Yuan Wang for technical support.

DISCLOSURES:

The authors have nothing to disclose.

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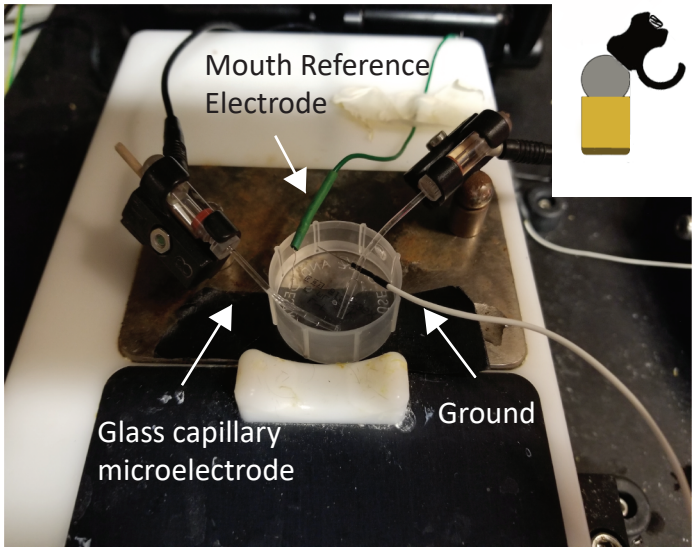
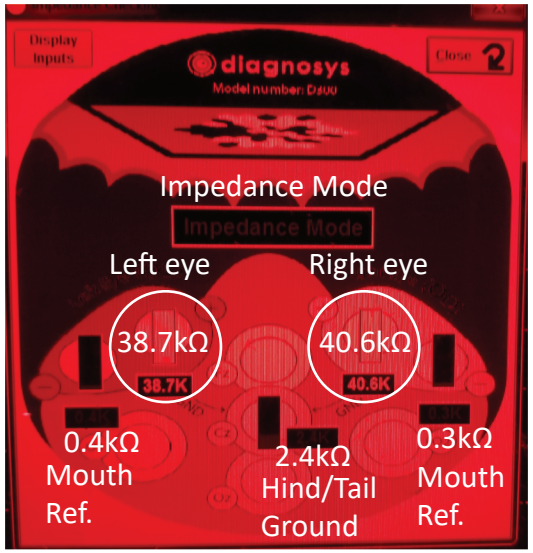
Figure 1

A



B



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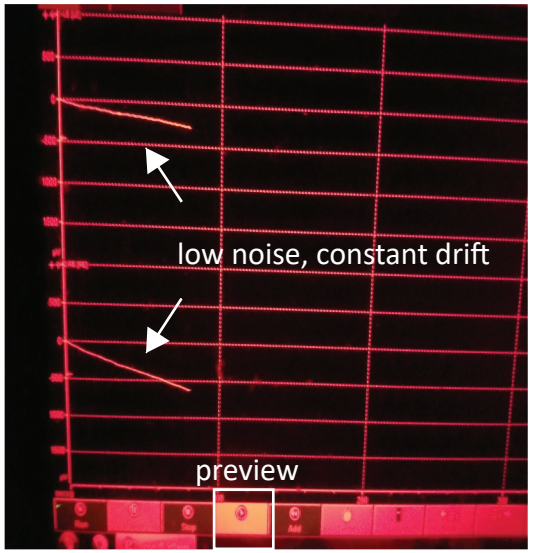

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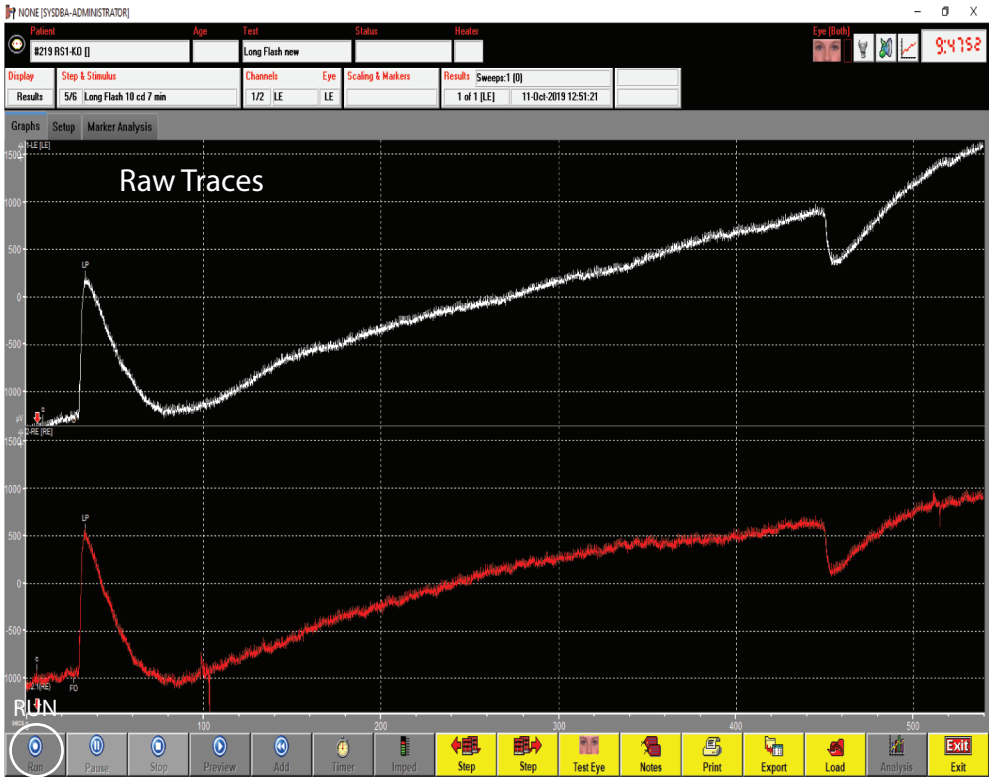

E

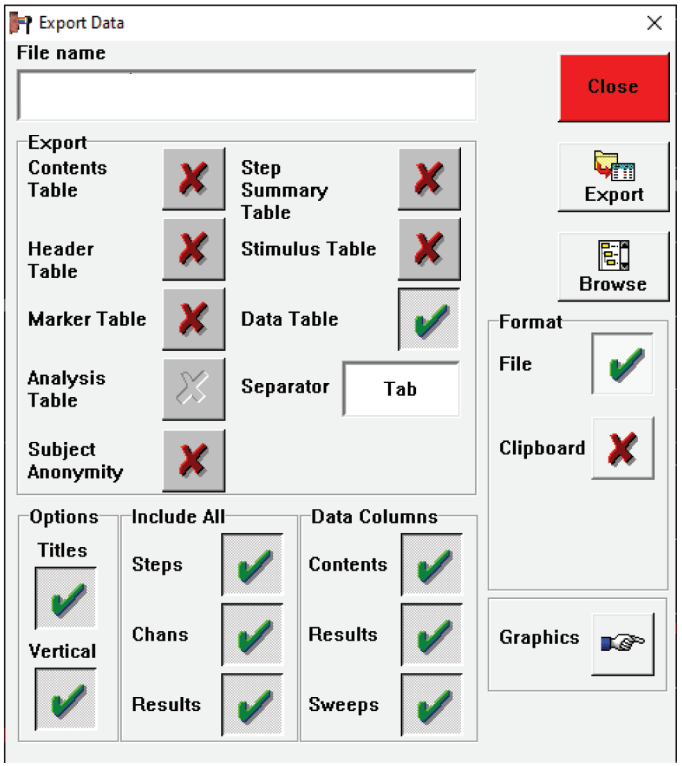

Figure 2

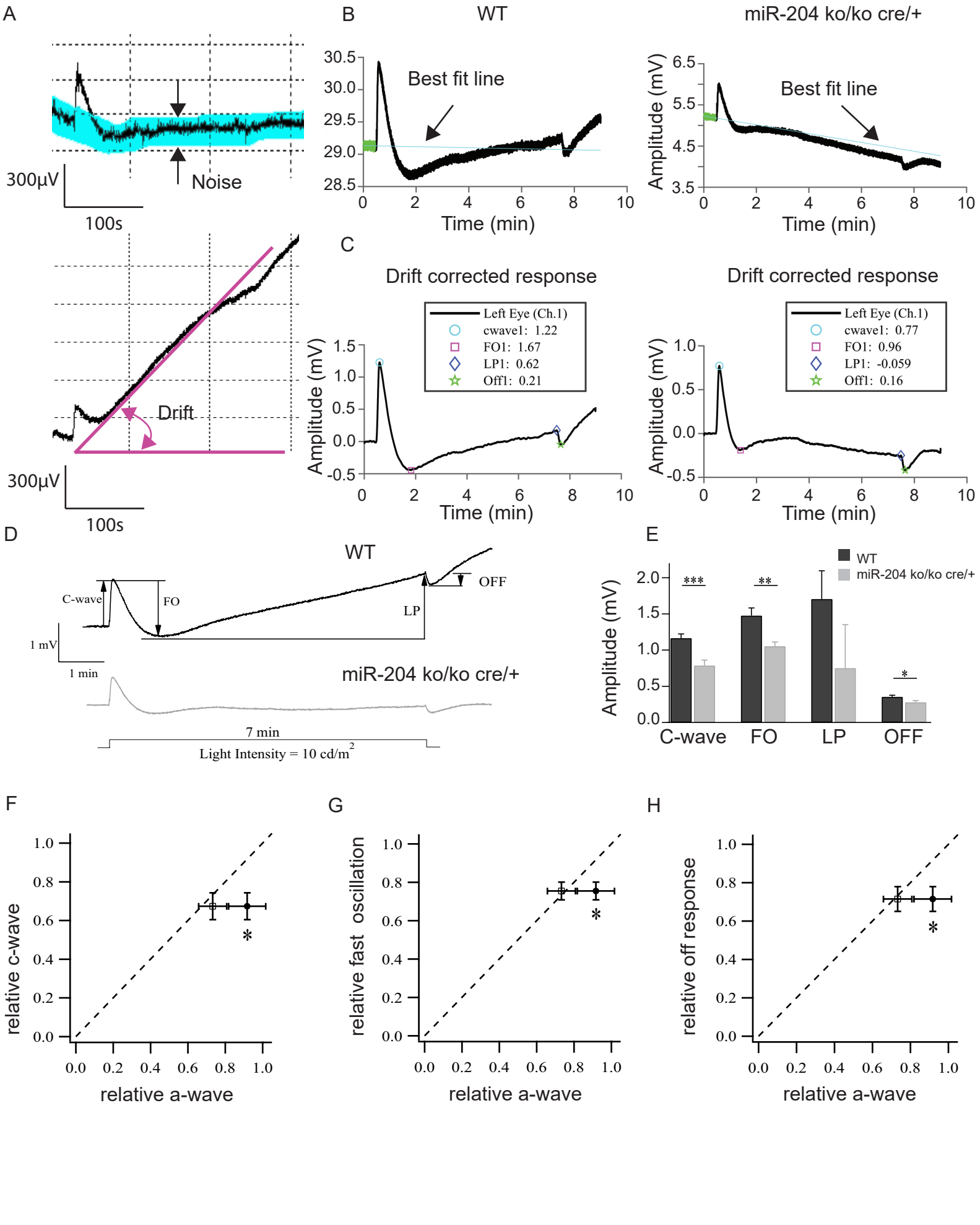

Table of Materials

Click here to access/download;Table of Materials;JoVE Table of Materials_05152020JM.xlsx

Name of Material/Equipment	Company	Catalog Number
Ag/AgCl (mouth) Electrode	WPI Inc	EP1
Ceramic Tile	Sutter Instrument	CTS
Cotton Tipped Cleaning Stick	Puritan Medical Products	867-WC No Glue
Electroretinogram (ERG) System	Diagnosys LLC	E3 System
Bunsen Burner	Argos Technologies	BW20002460
Glass Capillary Tube (1.5 mm)	Sutter Instruments	BF150-75
Hank's Buffered Salt Solution (HBSS)	Thermo Fisher Scientific Inc	14175-095
In-Line Filter	Whatman	6722-5001
Low Noise Cable for Microelectrode Holders	WPI Inc	5372
Magnetic Ball Joint	WPI Inc	500871
MatLab	Mathworks	
Matlab Curvefit Toolbox	Mathworks	
MatLab Compiler	Mathworks	
MatLab Runtime version 9.5	Mathworks	R2018b (9.5)
Microelectrode Holders (45 degrees)	WPI Inc	MEH345-15
Needle (25 ga)	Covidien	8881250313
needle (ground) electrode	Rhythmlink	13mm - one elctrode
Regulator/Power Conditioner	Furman	P-1800
Syringe (12 mL)	Monoject	1181200777
T-clip	Cole-Parmer	06852-20
Vacuum Desiccator	Bel-Art	420120000
Pharmacological treatment		
Lubricant eye gel	Alcon	0078-0429-47
Phenylephrine Hydrochloride 2.5%	Akorn	17478-201-15
Proparacaine Hydrochloride 0.5%	Akorn	17478-263-12
Tropicamide 0.5%	Akorn	17478-101-12
Xylazine	AnaSed	sc-362949Rx
Zetamine (Ketamine HCl)	VetOne	501072

Comments/Description

Mouth reference electrode for mouse

Used to cut the glass capillary tube to an appropriate size

To be used as a spacer to improve the fit of the electrode holder assembly

Visual electrophysiology system to diagnose ophthalmic conditions in vision research and drug trials

Or equivlaent to shape glass under flame

For filling with HBSS and making contact to the cornea

Commercially available. Maintain at RT

To protect vacuum pump from aerosols

Suggested for improving the length and placement of the cables and electrode holder assemblies

For magnetically positioning the electrode holder assembly on the stage

MatLab: For editing the analysis software

Toolbox for MatLab (only required for editing the analysis software)

Toolbox for MatLab (only required for editing and re-releasing the analysis software)

Required to run the analysis software: https://www.mathworks.com/products/compiler/matlab-runtime.html

For holding the capillaries

For filling the capillary tubes with HBSS

Subdermal needle electrode (ground) for mouse (13mm long, 0.4mm diameter needle, 1.5m leadwire)

Or equivalent to remove DC-offset from noise introduced through power line

For filling the capillary tubes with HBSS

For electrode holder assembly

Clear polycarbonate bottom & cover

Helps lubricates corneal surface and maintain electrical contact with capillary electrodes

Short acting mydriatic eye drops (for pupil dilation)

Local anesthetic for ophthalmic instillation

Short acting mydriatic eye drops (for pupil dilation)

Analgesic and muscle relaxant

Anesthetic for intramuscular injections

Dear Dr. DSouza,

We thank the reviewers for their constructive comments and suggestions that have allowed us to address specific concerns and provide further clarifications of the protocol and representative data. We believe that the revised manuscript has significantly benefited in terms of clarity and scientific rigor. As per the reviewers' suggestions, we elected to replace the RS1 KO mouse with a different mouse model with a known RPE defect (conditional knockout of miR-204 in the retinal pigment epithelium). The data presented is complementary (unpublished data) to a previous publication on miR-204 null mice (global KO model). Track changes has been removed since text outside of the basic protocol had to be completely rewritten. We also revised the data analysis section to include a new compiled version of the MATLAB script file that we included previously. This executable file can perform the DC-ERG data analysis without requiring programming knowledge or the need for a MATLAB license. We've also added the ability to export the measured DC-ERG values to an excel file. Below are the point-by-point responses that address each of the reviewer's comments.

Josh Miyagishima

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Spelling and grammatical errors have been corrected.

• **Introduction:** Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

An additional paragraph has been added to the introduction:

The retinal pigment epithelium (RPE) is a monolayer of specialized cells that line the posterior segment of the eye and exert critical functions to maintain retinal homeostasis [1]. The RPE supports photoreceptors by regenerating their photon-capturing visual pigment in a process called the visual cycle[2], by participating in the diurnal phagocytosis of shed outer segment tips [3], and in the transport of nutrients and metabolic products between photoreceptors and the choriocapillaris [4,5]. Abnormalities in RPE function underlie numerous human retinal diseases, such as age-related macular degeneration [6], Leber's congenital amaurosis [7,8], and Best vitelliform macular dystrophy [9]. As donor eye tissues are often difficult to obtain solely for research purposes, animal models with genetic modifications can provide an alternative way to study the development of retinal diseases [10,11]. Additionally, the emergence and application of CRISPR cas9 technology now permits genomic introductions (knock-in) or deletions (knock-out) in a simple, one-step process surpassing limitations of prior gene targeting technologies [12]. The

boom in the availability of new mouse models [13] necessitates a more efficient recording protocol to non-invasively evaluate RPE function.

• **Protocol Language:** Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Examples NOT in the imperative:
1) 4.1,4.2, 4.3, 4.4, 4.5, 4.6, 4.8 etc

Changes to the protocol have been made to ensure that it is written in the imperative tense.

• **References:** Please spell out journal names.

The Journal names have been manually typed out - JOVE has not submitted the updated required format to endnote (last update July 2017).

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Multifocal Diagnosys, Espion E3 System with ColorDome Ganzfeld Illumination, Vacu-Guard, GenTeal Gel,

1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

All commercial names have been removed from the manuscript and are referenced appropriately in the table of materials/reagents.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All data presented is unpublished.

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript# JoVE61491 entitled "Direct coupled electroretinogram (DC-ERG) for recording the light-evoked electrical responses of the mouse retinal pigment epithelium" by Miyagishima et al. is to be a JoVe produced video demonstrating the DC-ERG procedure on mice using the Espion Diagnosys system with tips for reducing noise and drift.

This procedure was pioneered for use routine use on rodents by Dr. Neal Peachey and Dr. Alan Marmorstein in the early 2000s utilizing a homemade ERG system adapted to measure the slowly-developing light-evoked potentials from the RPE. Use of their procedure in mice was published in detail in 2004. Here it seems that the authors want to provide their protocol files for use with the Diagnosys machine and demonstrate the procedure with the addition of how to make and optimize the electrodes. The authors do a nice job of providing a step by step guide for the procedure and including the protocol and analysis files, however, a few important concerns were identified:

1. The summary and abstract should explain that the methodology is not novel and was previously published using either a "home-made" ERG machine (Samuels et al 2010; Wu et al 2004) or the Diagnosys machine (Samuels et al 2015). Application of the improvement techniques to the making of the electrodes with the Espion Diagnosys machine is the novel part of the manuscript/video.

The authors thank the reviewer for their rightful assessment that the methodology was pioneered by the likes of Dr. Alan Marmorstein, Dr. Neal Peachey, Dr. Jiang Wu, Dr. Ivy Samuels, and others that developed the technique for use in mouse and rat models. Special acknowledgement of this fact is now included in the summary and abstract and additional references will be included in the introduction where it is described that this protocol is adapted from their previous work.

Summary: Page 0, Lines 33-34 "This technique was first described by Marmorstein, Peachey, and colleagues in the early 2000s."

Abstract: Page 1, Lines 48-50 "The methodology behind the DC-ERG was pioneered by Marmorstein, Peachey, and colleagues using a custom-built stimulation recording system [14,15] and later demonstrated using a commercially available system [16]."

Introduction: Page 1, Lines 85-87. "This protocol describes a method adapted from the work of Marmorstein, Peachey, and colleagues who first developed the DC-ERG technique [14-17] and improves upon the reproducibility and ease of use."

2. Figure 2A, it is unclear from the picture what the difference between noise and drift is. It would benefit to show the examples in separate windows.

We agree with the reviewer and now include two separate windows depicting noise and excessive drift in ***Figure 2A*** *(top panel, bottom panel).*

3. Line 224-225 Please provide an additional explanation for the statement "These defects in the retinal response are expected to be reflected in the electrical response of the RPE" as a reason to utilize the RS1 KO mouse for this manuscript. If the purpose is to show differences in RPE function, it seems more appropriate to use a mouse where the dysfunction is already published, or AT LEAST one where there is RPE degeneration or a known defect should be utilized as an example. Use of the RS1 mouse has given unclear results, that have not yet been published, and the authors also not seem to include an actual waveform trace from the RS1 mouse in the results. There are many mice with photoreceptor defects that do not present with abnormal RPE function. A mouse with altered RPE function such as the CFTR mutant (Wu et al 2006 PMID: 16626699) would be a more appropriate choice. Furthermore, any reduction in the c-wave or other dc-ERG components could be reflective of photoreceptor dysfunction. (See Ziccardi et al 2012 PMID: 22993419). The dc-ERG components should be normalized to the a-wave - and both should be shown in the manuscript/video.

The authors thank the reviewer for their suggestion to use a mouse model where RPE degeneration is a known defect. We have removed data pertaining to the RS1 KO mouse and have replaced it with a conditional KO of miR-204 in the RPE. There are several papers published on miR-204 and its essential role in maintaining the RPE epithelial phenotype along with its direct impact on Kir7.1 (inwardly rectifying potassium channel) expression [18,19]. Reduced KiR-7.1 expression on the apical membrane of the RPE will directly repress the RPE electrical response (c-wave). New figures are now provided that include the averaged a-wave and b-wave traces and the corresponding response intensity curves. Additionally, we now provide the DC-ERG traces for both WT and the KO. The amplitudes of the DC-ERG components are also included normalized to the a-wave amplitude.

4. For whatever mutant mouse is to be used, at least one representative dc-ERG recording from it should be shown, along with the corresponding strobe-flash ERG and a-wave analysis.

*As mentioned in 3. We have updated the figures to include a representative DC-ERG recording from the mutant and WT (****Figure 2B****, ****Figure 2C****, ****Figure 2D****) along with the corresponding dark-adapted strobe flash ERG (****Figure S1 A-E****) and a-wave analysis (****Figure 2F-H****).*

Reviewer #2:

Manuscript Summary:

The authors provide a protocol for recording the dc-ERG from mice using a commercial device that is available in many labs. The dc-ERG provides a useful assessment of RPE function, as the late ERG potentials arise reflect RPE activity. Although very few labs use this technique, providing the programming and analysis tools may allow that to change.

Major Concerns:

The inclusion of the retinoschisin mutant as an example mouse is strange. The RS1 protein is not expressed in the RPE and RPE dysfunction has not been attributed to the mutant not to retinoschisis patients. Thus the reduction of the dc-ERG components reflects the loss of photoreceptor activity that is ultimately the signal that drives this response (i.e., not light).

Recommend that this either be focused on wild type mice only, or (preferably) that a bona fide RPE mutant be incorporated. Such mutants re available from Jackson Labs, and have been published by Patsy Nishina.

We thank the reviewer for their comments regarding the RS1 KO mouse and have elected to replace this model with a conditional KO of miR-204 in the RPE. The RPE deficit of this mutant has been well established [18,19], and as the reviewer pointed out, provides a better example of the utility of the DC-ERG technique.

Minor Concerns:
Note that Alan Marmorstein co-developed the more recent use of dc-ERG to study mutant mouse models.

We are thoughtful for the reviewer's concern and have now made every effort to acknowledge the work of Marmorstein, Peachey, and colleagues in the Summary, Abstract, and Introduction for pioneering the DC-ERG technique to study mutant mouse models.

Reviewer #3:
Manuscript Summary:
The manuscript by Miyagishima et al. titled "Direct coupled electroretinogram (DC-ERG) for recording the light-evoked electrical responses of the mouse retinal pigment epithelium" gives a detailed description of experimental materials and protocols to measure in vivo DC-ERG responses to a step of light following method described earlier by Peachey et al. 2002 and Wu et al. 2004 with some minor changes that are expected to improve stability and quality of recordings. In general methods are described in detail and provide a detailed account that should allow those who have performed traditional in vivo ERG before to adapt their Diagnosys in vivo ERG system for DC-ERG recordings revealing something about the functional state of RPE cells in live mice. I have some critique and suggestion listed below that can hopefully improve the manuscript. Unfortunately, due to current pandemic, I'm not able to verify the details of Diagnosys software instructions and I do not have Matlab software installed for evaluation of the scripts provided in this manuscript.

Minor Concerns:
1. Authors could explain explicitly rationale and benefits for using glass electrodes filled with HBSS solution as compared to standard e.g. gold loop electrodes. Comparison data would help to demonstrate the necessity/benefits.

Glass electrodes filled with HBSS maintain lubrication of the mouse's eye and prevent corneal dehydration that would occur with the use of standard gold loop electrodes. This explanation will now be included in the text (Page 3, Lines 161-162).

2. This reviewer did not see instructions for preparing or purchasing HBSS.

The HBSS used is commercially available and is included in the materials list (including the catalog #). We will include a note in the text referencing the Table of Materials to make sure this is clear.

3. 3.5 does not seem to clarify the detailed filling method (perhaps it is in 3.6 but as 3.5 reads now is somewhat confusing).

We thank the reviewer for their request for clarity and have made the following edits to 3.5 to make this point clear.

Page 3, Lines 140-141: "3.2 Pour 30 mL of HBSS ***(Table of Materials)*** *into an open 50 mL conical tube and place it (with the cap removed) into the vacuum chamber."*

4. 4.5, could authors quantify what would be a normal/acceptable drift. How much this drift increases or does it stay the same when electrodes are connected to the mouse?

We appreciate the opportunity to address the reviewer's comments.

Page 4, Lines 206-211 have been added to section 4.5

"The amount of drift observed when the electrodes are placed in the HBSS bath is generally less than 500uV/80s once they have stabilized and is equivalent to the drift observed when the electrodes are connected to the mouse. Thus, the electrical readout of the electrodes in the HBSS bath are an important indicator of the status of the electrodes. The noise, measured as peak-to-peak, is generally ~ 10-15% greater in the mouse than in the HBSS bath. This is probably due to the addition of motion artifacts from breathing."

5. 3.5 - 3.7 refers to electrode holders but do not refer to anything in terms what they are. Detailed instructions or better magnified picture of the mounting system for electrodes would help.

We apologize for the lack of clarity. We now include a detailed schematic of the mounting system for the electrodes as an inset in Figure 1a and added a description to 3.7.

*Page 3-4, Lines 173-183: "3.7 Install and secure the microelectrode holder into the custom-made T-clip/Magnetic ball joint stand (****Figure 1A****, inset). The placement of the electrode holders and recording electrodes on the stage are facilitated by the use of a custom microelectrode holder stand that consists of a T-clip (5/16"-11/32" OD Tubing) #8 (****Table of Materials****) that has been modified by removing the black polyacetal clips on one side. The cylinder base of the magnetic ball joints (****Table of Materials****) are cut in half to adjust the height. The modified T-clips are secured to the magnetic ball mounting screws with M3 sized nuts. The microelectrode holder fits within the modified T-clip and is held tightly in place by sliding in approximately a 1-inch tapered wooden handle made from breaking a Cotton Tipped Cleaning Stick (****Table of Materials****) at an angle. This customized electrode holder stand utilizes a rare earth magnet that can be securely positioned on the metal plate on the stage and enables 360 ° rotation on a 180 ° axis. "*

6. Rationale for selecting RS1 KO mouse for this study is not clear. Wouldn't it be better to have a mouse model with an RPE-specific defect. The interdependence between light-evoked activity in the neural retina with RPE component is very complicated and complicates interpretations of the signal. Authors didn't show a- and b-waves for comparison measured in the same system.

*We thank the reviewer for their comments regarding the RS1 KO mouse and have elected to replace this model with a conditional KO of miR-204 in the RPE (see new **Figure 2**). The RPE deficit of this mutant has been well established [18,19], and as the reviewer pointed out, provides a better example of the utility of the DC-ERG technique. We also include the a-wave and b-waves for comparison measured on the same system (**Figure S1**).*

7. Is it possible to quantify how much the vacuum-treatment of HBSS decreased noise or drift or occurrence of air bubbles.

The occurrence of bubbles is greatly suppressed with the use of vacuum. Prior to making the changes detailed in our DC-ERG protocol our rate of obtaining a successful recording was ~ 25%. After making these changes our success rate has improved to > 75%. Previously the high failure rate was primarily due to bubbles accumulating in the electrode creating unwanted drift when the bubbles moved along the capillary or increased noise when they altered the impedance. Now the few failed recordings are almost all directly attributed to the health and status of the mouse under anesthesia (e.g. respiratory distress) or whether the eyes are even suitable for recording (e.g. prior visually significant cataract).

Reviewer #4:

The manuscript by Miyagishima and colleagues describes a method for obtaining DC-ERG recordings from mouse eyes for studying the light-evoked electrical responses generated by the RPE. While ERGs are widely used to study the electrical activity of the retina, primarily photoreceptors and bipolar cells, it is rarely applied for investigating the function of RPE cells. This is mostly due to the rather slow RPE response, compared to the neural retina's response, which necessitates prolonged light stimuli and long recordings prone to noise and instability. The authors describe in detail their recording method, with emphasis on preparing recording electrodes under vacuum to reduce bubbles. They also mention the use of voltage regulator/power conditioner to minimize power line artifacts. The authors provide an example of the type of data that can be generated by this method using wild type control and retinoschisin (RS1) knockout mouse, demonstrating the type of RPE phenotype that can be observed with DC-ERG recordings.

Overall, this is a worthy contribution that provides sufficient level of technical detail and experimental insight to serve as a reference to people interested in implementing this technique. Several issues should be addressed to improve the strength of the manuscript:

Substantive issues:

- Can bubbles be eliminated by the use of 1% Agar gel bridge in the recording electrodes instead of vacuum? This is a widely used method in suction electrode recordings that works well for preventing bubble formation.

This is an interesting idea brought up by the reviewer. I've used agar bridges quite extensively for suction electrodes recordings in the past. Although they themselves will tend to dry out with time, this could be overcome by storing them in HBSS between mice or while the conventional retinal ERGs are being recorded with the gold loop electrodes. However, there are two advantages to using HBSS. One advantage is that it prevents the cornea from dehydration. Even with the eye lubricants added prior to the recording the eyes still tend to dry out. Secondly the HBSS maintains the electrical connection by capillary action which can overcome slight motion (animal breathing) whereas the agar bridge would likely lose contact as the eye lubricant dries.

- Can the authors comment of the rationale for using 10 cd/m2 light intensity? Can other dimmer/brighter intensities also be used? Can additional information be extracted from the use of various light intensities?

We have previously estimated that 10 cd/m2 for 7min would bleach approximately ~ 10%-11% of the rod photoreceptors [19]. In isolated mouse rods a 10% bleach is expected to reduce the photocurrent by 50% [20], and notably decrease the potassium concentration in the subretinal space (e.g. 5mM to 1mM [21]).

We select 10 cd/m2 because in WT mice it evokes approximately half the size of the maximal response for all the components of the DC-ERG. [22] Of particular interest are the c-wave and fast oscillation of which the origins of these responses are well characterized and can then be isolated and studied further in invitro RPE models (e.g. iPSC-RPE). As the reviewer pointed out using other light intensities can extract additional information, for example the Off response undergoes a reversal of polarity at brighter light stimuli and may show differences at the point at which this reversal takes place. The user is free to change light settings at their discretion.

This information is added to the text on page 2, Lines 103-112.

- Can the authors comment on the rationale for using a step of light of 7 min duration over longer and shorter light stimuli? Can shorter light steps be used and why or why not?

We thank the reviewer for requesting an explanation and would like to draw their attention to a paper by Steinberg and colleagues where they used microelectrodes to perform in vivo, intraretinal and intracellular recordings of the RPE's electrical responses [23].

Here the authors' use 5 min of illumination and show that the apical membrane potential (Vap) tracks the change in K in the subretinal space. There is a rapid hyperpolarization as the K in the subretinal space drops (in response to photoreceptor activity) and then repolarizes as K reaccumulates to a new steady state in light. This corresponds with K as the main contributor to the c-wave. However, the basal membrane potential (Vba) is slower to hyperpolarize and the subsequent depolarization (attributed to the light peak substance) takes the entire 5 minutes to return to 90% of the baseline level (prior to light onset). To record the full extent of the light peak the DC-ERG recording would have to be long enough to allow for the full depolarization of the basal membrane which would be greater than 5 minutes. Thus, a 7 minute light step duration would be more than sufficient time to measure the Light peak for most mouse models where the light peak may be slower or faster than anticipated. Longer light steps do not add additional information and are also not practical because the length of the recording is already an obstacle.

- The abstract mentions the use of a voltage regulator/power conditioner to minimize power line artifacts and noise. However, this issue is not discussed other than being mentioned in the Modification and Troubleshooting section. Can the authors demonstrate how the quality of the recordings is affected by a power conditioner by providing an example of recordings with and without its use?

The voltage regulator/power conditioner is most useful at eliminating large DC offsets introduced to the baseline of the recording by the switching of power supplies in adjacent rooms.

We have now included an example of a recording where this takes place with and without the power conditioner in place (Supplementary Figure 2). The initial spike is still visible even with the power conditioner, but the DC offset is removed enabling the components of the DC-ERG to still be measured.

Minor issue:
- Page 3, line 170: For consistency with the rest of the section, "A drop of GenTeal Gel was placed…" should probably be "A drop of GenTeal Gel is placed…"

We thank the reviewer and have made the suggested editorial change under the Journal's policy to have the procedures written in the imperative:

(Page 5, Lines 236-237) "Place a drop of lubricant eye gel on each eye to maintain conductivity and prevent desiccation during the recording. "

Reviewer #5:
Manuscript Summary:
This manuscript describes a protocol to quantify changes in c-wave function which are a direct reflection of the RPE function. The protocol is described in sufficient details for an individual with basic knowledge of electroretinography to follow.

Major Concerns:
This reviewer has no major concerns.

Minor Concerns:
The text would benefit of some grammar editing to improve the clarity of the text.

We thank the reviewer and have proofread the manuscript for grammatical errors in an effort to improve the clarity of the text.

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Figure S1. ERG responses of WT and miR-204 ko/ko cre/+ mice.

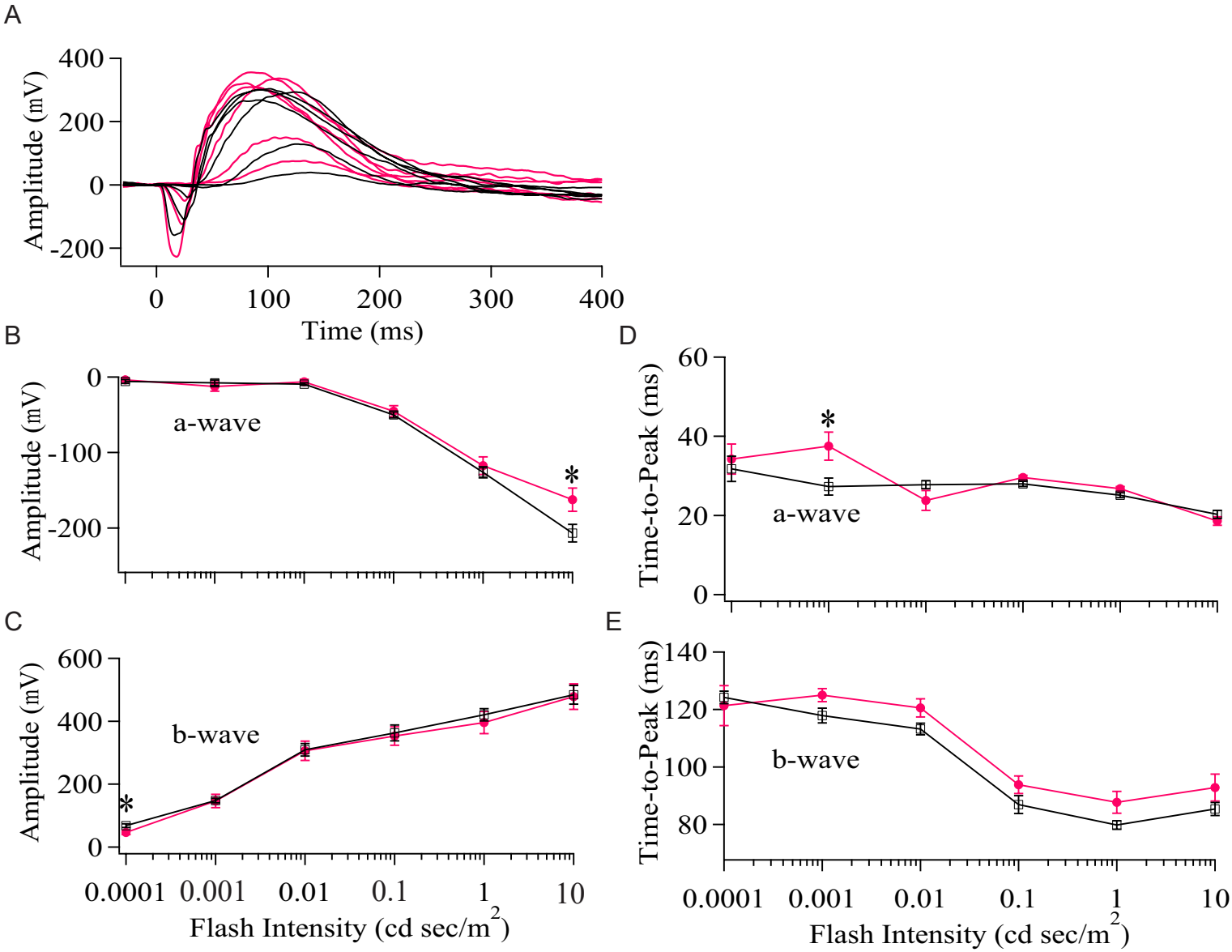
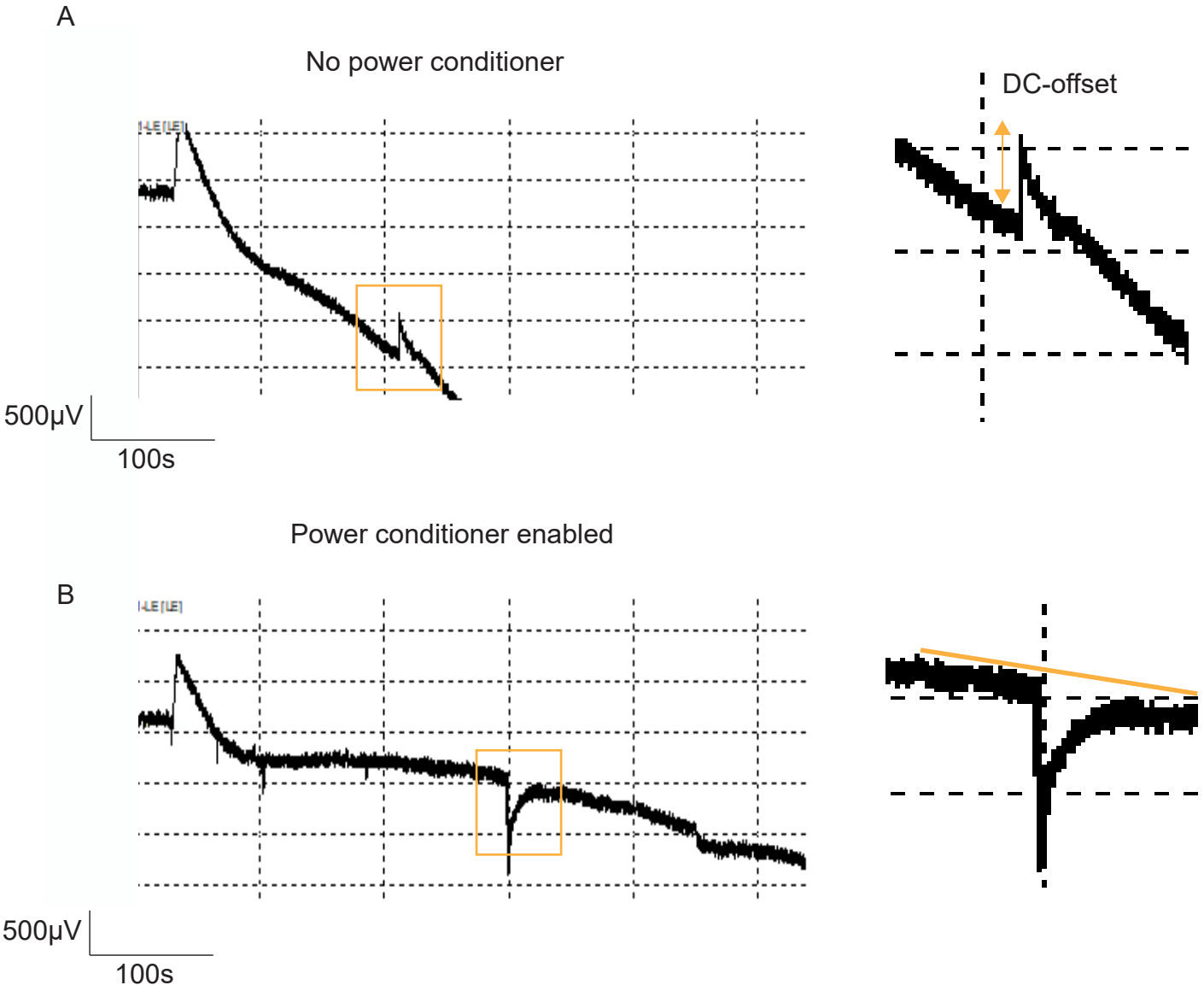
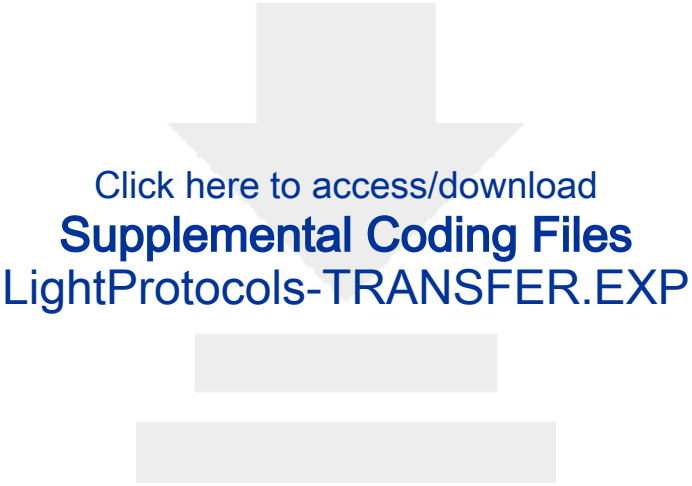


Figure S2. Example of interruption of DC-offset in power line mitigated by power conditioner



Suppl File 1



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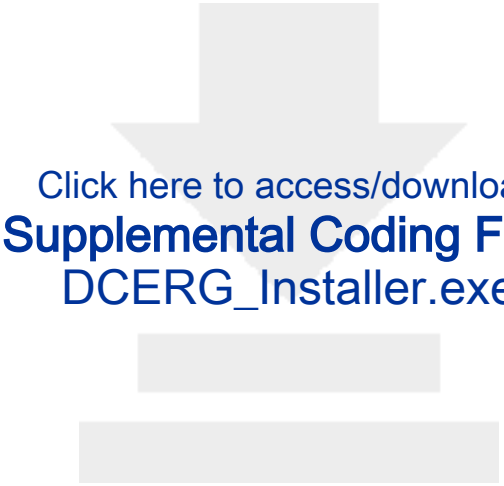

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DCERG_Installer.exe

fig 1

fig 2

Supplemental fig1

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