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An inexpensive device for non-invasive electroretinography in small aquatic vertebrates

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Abstract

Electroretinographic (ERG) method records a sum field potential of the retina in response to light. It mainly arises in the outer retina and is used as a non-invasive measure in both animal experiments and the clinic. Since it is a comprehensive method to assess outer retinal function, it is becoming increasingly useful in genetic studies of vision. Here we present a simple in-house built setup to measure ERGs of aquatic vertebrates. We have used this setup to efficiently and reliably measure intact larvae of zebrafish (*Danio rerio*), Medaka fish (*Oryzias latipes*), and *Xenopus laevis* tadpoles. By slight modification of the setup, we were also able to measure adult zebrafish and Medaka, demonstrating the general versatility of the setup. We picked these organisms since they are increasingly used to study visual function with genetic means. This setup is easily built and will be particularly useful for laboratories setting up ERG measurements as a complement to their genetic studies.
1. **Introduction:**

The electroretinogram (ERG) is a comprehensive and non-invasive method to probe outer retinal function. It measures light-induced changes of electrical activity of the eye in response to light. These sum field recordings are generally measured with an extracellular recording electrode placed on the cornea or in the vitreous chamber of the eye. In the vertebrate retina photoreceptors, bipolar and Müller glia cells are arranged in parallel, so that radially directed extracellular current flows of these cell types sum up.

The vertebrate ERG originates in the outer retina and features three prominent waves, an initial negative a-wave, reflecting photoreceptor activity, a large positive b-wave reflecting mainly ON-bipolar cell activity, and the d-wave reflecting postsynaptic activity involved in the OFF response (Dowling, 1987). The ERG is therefore an elegant method to assess outer retinal responses to variable light stimuli and adaptation conditions. It is used extensively in both basic research and ophthalmic practice.

Recent years have seen a rekindled interest in the use of aquatic vertebrates and their larvae in vision research. This is partly due to an increasing focus on using small aquatic vertebrate larvae in genetic experiments. For instance forward genetic screens in the zebrafish (*Danio rerio*) have uncovered a large collection of mutant strains that produce larvae with visual deficits, some of them closely mirroring human diseases (Malicki, 2000; Bilotta and Saszik, 2001; Goldsmith, 2001; Neuhauss, 2003). This collection of zebrafish strains with visual defects will be an entry point into the genetic dissection of vertebrate visual system development and function.
The ERG is ideally suited to assess outer retinal function in visually impaired animals in order to locate genetic defects in the visual pathway. Lack of an ERG response argues for a defect in photoreceptors, altered traces may be associated with synaptic defects in the inner retina, and normal recordings argue for a defect beyond the first photoreceptor synapse (Brockerhoff et al., 1995; Neuhauss et al., 1999).

Here we describe a simple, reliable, and low-cost ERG setup. We successfully tested our setup on intact aquatic larvae of three species: zebrafish (*Danio rerio*), medakafish (*Oryzias latipes*), and the African clawed frog (*Xenopus laevis*). These species were selected for their laboratory use as genetically modifiable model species. With only slight modifications in the animal preparation (Bilotta et al., 2001), ERGs from adult aquatic species can also be obtained, as we show for both adult Medaka and zebrafish. To our knowledge this is the first report of non-invasive ERG measurements from intact Medaka retinas and one of the few from intact Xenopus retinas.
2. Materials and Methods

2.1. Animals

Adult zebrafish (*Danio rerio*) of the WIK inbred strain were maintained and bred as previously described (Mullins et al., 1994). Larvae were staged by days after fertilization (dpf) at 28°C in E3 medium (in mM: 5 NaCl, 0.17 KCl, 0.33 CaCl$_2$ and 0.33 MgSO$_4$). Larvae were measured at 5dpf in the afternoon.

Medaka (*Oryzias latipes*) of the inbred CAB strain were raised and bred as previously described (Yamamoto, 1956). Embryos were raised at 28°C in ERM (embryo rearing medium: (in % (w/v) 0.1 NaCl, 0.003 KCl, 0.004 CaCl$_2$, 0.016 MgSO$_4$) and measured as young fish at 12 dpf in the afternoon. Stages were determined according to (Iwamatsu, 1994).

Tadpoles (*Xenopus laevis*) were kept in 0.1× Marc’s Modified Ringer solution (MMR (in mM): 10 NaCl, 0.2 KCl, 0.1 MgCl$_2$, 0.2 CaCl$_2$, 0.5 Hepes, pH 7.5). Tadpoles were tested at stage 42 (98 hours post fertilization) in the morning. Larvae were staged according to the normal table of (Nieuwkoop and Faber, 1975).

Adult medaka and zebrafish of both sexes ranging in size from 2-3 cm in length were used. All experiments were performed at room temperature.

2.2. Stimulation

A one channel optical system was used to present visual stimuli. White light was presented over the subject’s eye through the channel, which fitted with a 24V 250W projection lamp (Liesegang Diafant 250, Düsseldorf, Germany) as its light source.
The light from the beamer was collimated, passed through a neutral density filter wheel and focused onto a mechanical shutter head. A light guide was used to deliver light over the animal’s head. Both ends of the light guide were fitted with diffusers (Fig. 1). The optical system was outside a Faraday cage. The shutter was controlled by software under Windows OS environment (A.M.P.I. Master-8, Jerusalem, Israel). Stimulus intensity was controlled using a neutral density filter wheel. Unattenuated light intensity over the subject’s head was 3100 lux (optical density, OD is equal to 0 log unit), as measured by a light meter (Tektronix J17).

2.3. Recording Setup

Recordings and data acquisition were performed by an in-house built electroretinography setup, with the recording setup inside a grounded Faraday cage (Fig.1). ERG recordings were performed using an Ag/AgCl micropipette holder with a silver wire (MEH3SW10 WPI, Sarasota, CA USA) as recording electrode. The chlorodized wire was inserted in to a glass micropipette with a tip opening of approximately 20 µm. The pipette was filled with E3, ERM or MMR medium respectively. Reference electrode was an Ag/AgCl pellet assembly (HLA-003, Axon Instruments, Union City, CA USA) beneath the subject’s body, separated by a piece of wet tissue paper.

The electrical signal from both electrodes was differentially amplified 1000 times with a band pass between 3Hz and 100 Hz. A system of pre-amplifier--amplifier and filters was constructed in-house. Recording signals from the amplifier were sent to a 40 MHz dual channel storage oscilloscope HM 408 (Hameg GmbH, Frankfurt am Main, Germany) and to a DAQ Board NI PCI-6035E (National Instruments, Ennetbaden, Switzerland) via NI BNC-2090 accessories.
2.4. Experimental Procedure

All pre-recording steps were done under red illumination to minimize bleaching of the visual pigment. Preparation and recordings were performed in a tight Faraday cage under visual control using an upright microscope (Zeiss Stemi SV8, Oberkochen, Germany). Larvae and tadpoles were placed on a piece of a moistened sponge in a 35 mm Petri dish. The subjects were dark-adapted for at least 30 minutes prior to positioning them in the recording chamber. For larval recordings, each larva or tadpole was placed on its side on the surface of the moist sponge. Tadpoles were anesthetized with 0.02% buffered 3-aminobenzoic acid methyl ester (MESAB; Sigma-Aldrich, Buchs, Switzerland) in MMR medium. Zebrfish larvae and young Medaka fish were paralyzed by directly adding a droplet of the muscle relaxant Esmeron (0.8 mg/ml in larval medium; Organon Teknika, Eppelheim, Germany) onto the animals. The electrode was positioned in the approximate center of the cornea. Since the tadpole head is covered by a thin transparent tissue (the pellucida), a small incision had to be made to gain direct access to the cornea.

For adult recordings the setup was slightly modified similar to (Saszik et al., 1999). Briefly, the recording electrode was inserted into the vitreal chamber of the eye through an incision in the cornea. A chlorodized silver wire was fixed on the opposite nostril as a reference electrode. Normal fish water was taken to prepare a medium containing 0.01% buffered 3-aminobenzoic acid methyl ester (MESAB, Sigma-Aldrich, Buchs, Switzerland) and 0.1 mg/ml Esmeron (Organon Teknika, Eppelheim, Germany).
In order to ensure oxygenation of the animal, the medium was flushed by gravity forces over the gills by a plastic tube inserted into the mouth. The flow rate was approximately 1 ml/min.

Animals were dark-adapted for at least 30 minutes before mounting the electrode under dim red light. Before exposure to light animals were adapted in complete darkness for at least 2 minutes. For photopic measurements, the subject was light-adapted for at least 2 minutes prior to measurement. Background illuminance was 60 lux. A 1 sec stimulus was chosen to separate ON and OFF response with an interstimulus interval of 5 sec. Illumination was increased in 1.0 log unit steps over the range from –5 log unit to –1 log unit.

2.5. Analysis

A virtual instrument (VI) under NI LabVIEW 5.1 was developed to use in all experiments. Amplified analog signals were sampled by means of NI PCI 6035E DAQ board connected to a NI BNC-2090 BNC terminal block. Sampling was done in buffered acquisition mode with a sampling rate of 500 Hz. Recording was triggered by the shutter signal. To analyze the ERG response with respect to the actual onset of the light stimulus, mechanical shutter delay was measured by means of a photodiode. Traces were normalized to the baseline by subtracting the average potential before the stimulus onset. Responses were averaged between 3 and 7 times depending on the signal to noise ratio.

Statistical analysis was performed using SPSS v.11 (SPSS Inc., Chicago, USA) and graphs were generated using Origin v.7.0 (OriginLab, Northampton USA).
3. Results

3.1. Electroretinogramms

In order to test our setup, we first measured zebrafish larvae at 5pdf, since a number of studies have published ERG recordings at this stage (e.g. Brockerhoff et al., 1998; Saszik et al., 1999; Seeliger et al., 2002). We measured larvae under both dark and light-adapted conditions with a stimulus of one second duration in order to temporally separate ON and OFF responses (Fig. 2). In the dark-adapted larva, already at the lowest light intensity a positive deflection, the b-wave, appears. At increasing light intensities the b-wave rises in amplitude and the OFF response (d-wave) appears (Fig. 2A). The b-wave usually occludes the a-wave, which is therefore only occasionally visible. Pharmacological blocking of the b-wave can reveal the negatively reflecting a-wave, which also increases in amplitude with increasing light intensity (data not shown). In general the ON response under light adapted conditions is less sensitive, but similar in waveform (Fig. 2B). The recorded ERGs are typical for vertebrates and conform to published reports of the zebrafish ERG using a similar stimulation paradigm (Seeliger et al., 2002).

3.2. System performance

A critical feature of any measuring device is the signal to noise ratio (SNR). The main source of noise in our system stems from interference from the building’s power installation and can be effectively controlled by a tight Faraday cage. Typically noise was below 10 µV, while recorded signals ranged from 20 to 1000 µV. Hence the SNR was lower at lower light intensities, making averaging necessary. Although at high light intensities no averaging was necessary, we generally averaged 3 to 7 traces at all light intensities.
We observed a variation in b-wave amplitude among larvae measured under the same conditions. This variation may be due to biological variation or stem from our setup. Hence we addressed this question by measuring the b-wave amplitude of 10 dark-adapted zebrafish larvae at a given illumination (-3 log). Each individual larva was measured three times after remounting the electrode in between trials. A one-way ANOVA analysis for repeated measurements revealed that most of the variation is due to inter-individual differences ($F=411.32; p<0.001$) rather than to variation between trials ($F=0.70; p>0.5$). There was also no significant correlation of b-wave amplitude and order of measurement, indicating that remounting the electrode did not influence subsequent measurements. Taken together, these data indicate that the described setup is reliable and variation is only marginally influenced by the mounting procedure.

3.3. Comparative Recordings from aquatic vertebrates

Having successfully tested our ERG device in zebrafish, we next set out to test other aquatic vertebrate larvae and adult fish. An important consideration is the oxygen supply of the retina during measurement. In small larvae, surface exchange should suffice, while in larger animals water has to be flushed over the gills to ensure proper oxygenation.

We measured young Medaka fish at 12 dpf, a stage where all animals have hatched. The resulting ERG is similar to 5 day-old zebrafish larvae (Fig. 3B). The ON response kinetics is slightly faster, probably reflecting the more matured state of the Medaka retina. We also recorded ERGs from stage 42 Xenopus tadpoles, a stage where rods and cones are histologically distinguishable. At this stage the pellucida, a transparent
tissue covering the eye, has to be penetrated by the electrode to gain access to the cornea.

ERGs from adult zebrafish (Fig. 3D) and Medaka (Fig. 3E) are also easily measurable and are comparable to larval recordings. The measurements tend to be more stable, reflecting the mature state of the visual system.

Having established that the tested aquatic animals display a typical vertebrate ERG, we compared the b-wave amplitude of dark-adapted animals (Fig. 4). The amplitudes differ remarkably little between larvae and adult animals.
4. Discussion

In the present study we report on a simple setup for electroretinographical recordings of vertebrate aquatic animals and their larvae. Such a setup allows a quick and informative survey of outer retinal function, as will be especially useful in assessing visual defects in genetically modified animals. The experimental setup can be readily and cheaply assembled, giving experimental results comparable to commercial solutions, usually tailored to the use in humans. Our recordings showed a good signal to noise ratio, so that in most cases averaging was not necessary. As a measure of the reproducibility of ERG recordings, we measured the inter-experimental variance in a number of zebrafish larvae and showed that the inter-individual variability is much higher than the variance between repeated measurements of the same animal. Hence the variance in ERG measurements is mainly due to biological variation.

Having established that zebrafish ERGs can be reliable recorded in our custom made setup, we set out to apply the technique to other aquatic vertebrate species. Since aquatic vertebrate species are increasingly used for genetic manipulation, we focused on species that are already used as genetic model organisms to study vision. A number of reverse and transgenic approaches have been used to study eye development in Xenopus. Recently these tools have also been employed to study functional aspects of vision, for instance by transgenic manipulation of photoreceptor properties (Wiechmann et al., 2003). The Medaka is a teleost species with favorable genetic properties (reviewed in Wittbrodt et al., 2002) that will likely play an increasingly important role in studies of vertebrate visual function. Finally, the
zebrafish is already extensively used in our and a number of other laboratories in genetic studies of visual function, including electroretinography (reviewed in Neuhauss, 2003).

We were able to record ERGs with our simple setup in all species, including adult zebrafish and Medaka. To our knowledge this is the first report of electroretinography in the Medaka and one of the few using an intact larval preparation in Xenopus. Most ERG studies using aquatic animals measure in eye cup preparations. Although in most cases the individual recorded eye is further used for histological assessment, the option to have the specimen survive the measurement may be of importance. Survival of the specimen allows repeated measurement, as we have used in this study, enabling comparison of retinal responses before and after experimental treatment. Furthermore, electroretinography may serve as decisive criteria which fish to propagate, for instance after screening adult fish for carriers of dominant founder mutations.

Our setup is easily adjustable to other species and will mainly involve ensuring proper oxygenation of the animal. Any larva below a length of one centimeter will likely be small enough to be sufficiently oxygenated by passive diffusion though the skin. In larger animals oxygenation through the gills is the method of choice with the added advantage that gill flow can be utilized to expose the animal to pharmacological agents. This simple and cheap setup should be suitable for undergraduate teaching and might be utilized by laboratories studying foremostly the genetics of vision without a special physiological focus.
Figure Legends

Figure 1. Electroretinography (ERG) setup for aquatic larvae. See text for details

Figure 2. Typical larval zebrafish ERG recording. Intensity series obtained under dark-adapted (A) and light-adapted (B) conditions at 5 dpf. The small a-wave (upward slanted arrow), the prominent b-wave (horizontal arrow), associated with the ON response and the d-wave (downward arrow), associated with the OFF response are apparent. Light intensity is in log units of illuminance.

Figure 3. Comparison of ERG waveforms of various aquatic larvae and animals. Dark adapted ERGs were recorded at –3 log unit of unattenuated light intensity. (A) 5 dpf zebrafish larva, (B) 12 dpf Medaka fish, (C) stage 42 Xenopus tadpole, (D) adult zebrafish, (E) adult Medaka.

Figure 4. Comparison of dark-adapted b-wave amplitudes over log intensity. Statistical evaluation over n number of animals. ◊, larval zebrafish, n=11; △, young Medaka, n=6; ▽, Xenopus tadpoles, n=8; □, adult zebrafish, n=6; □, adult Medaka, n=5. Error bars are MEAN±SEM.
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Figure 1
Figure 2
Figure 3
Figure 4