

# Chapter 2

## Anatomical, Physiological, and Behavioral Analysis of Rodent Vision

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### Abstract

This chapter provides protocols for the study of rodent vision. An advantage of the visual system is that the physiological and behavioral response to the natural stimulus, light, can be measured. Moreover, the anatomy and circuitry of the system have been the subject of much research. Here, we describe our protocols for the analysis of the distribution of neurotransmitter receptors and signaling molecules in the retina by immunohistochemistry. We also explain in detail how we record the electroretinogram from mice, and we review two behavioral tests of rodent vision. One, the virtual optomotor test, makes use of the optokinetic nystagmus reflex, and thus is simple to perform and does not require training. The other, the visual water maze, is more demanding but provides a true quantitative readout of vision performance.

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### 1. Introduction

As stated by John Dowling in 1987, the retina is an approachable part of the brain (1). The retina has a highly organized structure, being divided into five stratified layers, three cellular and two synaptic (**Fig. 2.1**). The light-sensitive photoreceptors that transduce light into an electrical signal are located in the outermost layer. The cellular and synaptic layers beneath the photoreceptors are collectively known as “neural retina” which is considered part of the central nervous system (CNS). The light response undergoes substantial signal processing within the neural retina before exiting the eye via the optic nerve en route to the

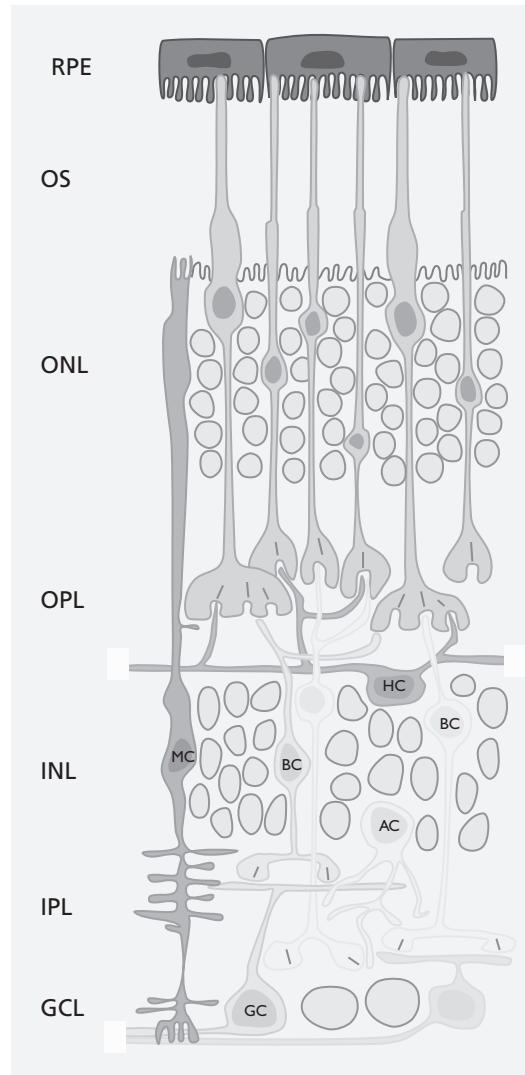


Fig. 2.1. Schematic diagram of the retina. The retina has a laminar structure. Photoreceptors (whose cell bodies are located in the outer nuclear layer; *ONL*) capture photons and transduce light into neural signals in their outer segments (*OS*). Retinal is regenerated and *OS* supported by retina pigment epithelial (*RPE*) cells. Neural signals are processed in the inner retina composed of two layers of cell bodies (the inner nuclear layer [*INL*] and the ganglion cell layer [*GCL*]). The *INL* comprises bipolar (*BP*), horizontal (*HC*) and amacrine (*AC*) cells. Muller glial cell (*MC*) soma are also located in the *INL*. The *GCL* comprises displaced amacrine and ganglion (*GC*) cells. Processing occurs in two synaptic or plexiform layers (the outer and inner plexiform layers [*OPL* and *IPL*, respectively]).

brain. The neurons within the neural retina are essentially identical to those within the brain: these neurons use most of the same neurotransmitters and neuropeptides and express many of the same receptors, ion channels, and enzymes as those found in the

brain. Therefore, the neural retina is a useful system to examine the effects of genetic mutations and pharmacological compounds on CNS function. The anatomy, physiology, and neural circuitry of the retina are well characterized, and the effects of drugs on specific neural pathways as they process light stimuli may be tested.

Rod photoreceptors and the peripheral retina which is rod dominated are well conserved across mammalian species. In contrast, cones and the central retina differ greatly between primates and other mammals. Humans, apes, and old world monkeys have a cone-rich macula and a central cone-only foveola that enables high visual acuity. Other mammals lack this specialized macula although the retinas of some mammals have a visual streak, an area of increased photoreceptor density and therefore, acuity, across the central meridian. Additionally, the cones of human and non-human primates contain one of three opsins; other mammals have one or two cone opsins. Here, we will discuss methods as they apply to rodent vision, particularly the rod system, and we will mention briefly when differences are necessary for studying humans.

In the first part of this chapter, we will provide methods to analyze the distribution of ion channels, neurotransmitter receptors, and signal transduction proteins by immunohistochemistry. In the second part of this chapter, we will present methods to examine the electrophysiological responses of the eye to light stimuli. Finally, we will describe two behavioral tests used to measure vision in rodents: the Visual Water Task is a task used to examine visual acuity, similar to that measured in clinic, and the Virtual Optomotor System, which relies on a visually driven tracking reflex.

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## 2. Materials

### 2.1. Immunohistochemistry

#### 2.1.1. Aqueous Mounting Medium (AMM)

1. Add 2.4 g Mowiol 4–88 (Hoechst) or Gelvatol 20–30 (Monsanto) to 6 g glycerol in a 50-ml conical tube. Stir to mix.
2. While stirring, add 6 ml distilled water and leave for 2 h at RT on a rocker.
3. Add 12 ml 0.2 M Tris (pH 8.5).
4. Add  $\text{NaN}_3$  to a final concentration of 0.02% (optional).

5. Incubate the tube at 50°C for 10 min or until Mowiol is dissolved.
6. Clarify by centrifugation at 5,000 *g* for 15 min. Store 1-ml aliquots in microcentrifuge tubes at –20°C.
7. Warm tubes to room temperature for use. Opened tubes can be stored at 4°C for approximately 1 month. Discard if any crystalline material is seen in the tube or on the slides.
8. For fluorescence, add 1,4-diazobicyclo-(2.2.2)-octane (DABCO) to 2.5% to reduce fading. This is a free radical scavenger. *Note:* since this is an aqueous medium fluorescence quality can diminish overtime. It is best to image 1 day after tissue is coverslipped.

### 2.1.2. Paraformaldehyde Fixative (PFA)

1. Heat 10 ml 0.1 M phosphate buffer, pH = 7.4 (PB) to 60°C in a microwave.
2. In a fume hood, for a 4% solution, add 0.4 g paraformaldehyde. *Caution:* paraformaldehyde fumes are toxic.
3. Stir until dissolved. If powder does not dissolve, add a drop of 5 N NaOH.
4. Chill on ice. Filter and adjust pH to 7.4 if necessary. Keep for about 1 week at 4°C.

### 2.1.3. Antibody Incubation Solution (AIS)

1. 3% (v/v) normal horse serum. (Sometimes less background is obtained by substituting normal goat serum.)
2. 0.5% (v/v) Triton X-100.
3. 0.025% (w/v) NaN<sub>3</sub> in phosphate buffer saline (PBS).

## 2.2. ERG Recording: Equipment

1. ERG Recording System with Ganzfeld. There are several commercial ERG recording systems that include hardware/software and a Ganzfeld dome, e.g., Espion E<sup>2</sup> (Diagnosys, Lowell, MA), UTAS (LKC Technologies, Gaithersburg, MD), RETI-port (Roland Consult, Brandenburg, Germany). (*See Note 1.*)
2. Optometer for measuring flash intensity and background intensity. Commercial optometers/radiometers include the IL1400A (International Light Technologies, Peabody, MA) and the S350/S450 (UDT, San Diego, CA). Both have an integration mode that enables the measurement of light from a brief flash.
3. A heating system to maintain core body temperature of the mouse between 36.0 and 37.5°C while the animal is anesthetized (*see Note 2*). Maintaining core body temperature is crucial for both animal health and obtaining maximal ERG amplitude, which drops dramatically with low body temperature. Loss of just a few degrees of body temperature is associated with reduced ERG amplitudes

and prolonged latencies. A simple solution is to place the animal on a warmed sports injury heat pad (2). Commercial systems include a low voltage DC system that enables automated temperature control (Braintree Scientific, Inc., Braintree, MA) and water-circulated heating systems (Roland Consult, Brandenburg, Germany). (*See Note 3.*)

4. A system for measuring core body temperature (if a non-automated system is used). We use the Thermalert temperature monitoring system with an RET-3 rectal probe for mice (Braintree Scientific, Inc., Braintree, MA).
5. A system for visualizing the mouse eye in the dark. We use a simple headlamp for most work in the dark. For fine manipulation work such as connecting electrodes we use an OptiVISOR binocular headband with LX-7 lens (2.75 ×) and an attached VisorLIGHT (Donegan Optical Company, Inc., Lenexa, KS). Two layers of deep red film are taped over both light sources and any lighted equipment in the room (e.g., computer monitor and green LED's used as ON lights on most equipment).

### **2.3. Medical Supply List for ERG Recordings**

1. Butterfly needle infusion set (25 ga × 3/4" with 12-inch tubing; Abbott Laboratories, North Chicago, IL).
2. Ketamine (100 mg/ml; Vedco, St Joseph, MO).
3. Xylazine (20 mg/ml; Lloyd Laboratories, Shenandoah, IA).
4. Saline solution (0.9% NaCl for injection; Hospira, Inc., Lake Forest, IL).
5. Sterile mix bottle (10 ml).
6. Murocel (1.0%; Bausch and Lomb, Tampa, FL).
7. Phenylephrine (2.5%, store at 2–8°C; Bausch and Lomb, Tampa, FL).
8. Tropicamide (1%; Akorn, Buffalo Grove, IL).
9. Proparacaine (1.0%, store at 2–8°C; Akorn, Buffalo Grove, IL).
10. Erythromycin ophthalmic ointment (5 mg/g, E Fougera & Co, Melville, NY).
11. Oxygen tank.
12. General supplies, including insulin syringes (0.5-ml 28 ga), 1-ml tuberculin syringes, fine forceps, tape, KY petroleum jelly, alcohol swabs, cotton-tipped applicators (Q-tips).
13. Miscellaneous equipment: Kitchen cooking scale for weighing mice in the laboratory, aluminum foil for making electrodes shields, tooth brush, and nail file for cleaning electrodes.

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### 3. Methods

#### 3.1. *Immunohistochemical Analyses*

Our laboratories are interested in localizing molecules involved in retinal synaptic transmission. We perform immunofluorescent labeling of retinal cryosections, since this allows the localization of multiple antigens. Importantly, we have found that short fixation times are essential for preserving antibody staining of synaptic membrane proteins, such as receptors or ion channels. While obtaining lightly fixed human retinal tissue can be problematic, we will describe here our procedure using mice.

##### 3.1.1. *Tissue Preparation*

1. Mice are euthanized using an overdose of isoflurane or pentobarbital delivered via intraperitoneal injection.
2. Eyes are rapidly enucleated with curved scissors (care must be taken to not damage the optic nerve otherwise the retina will pull away from the eyecup during dissection), and rinsed in cold 0.1 M PB with 2 mM  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  added.
3. Eyecups are prepared by cutting just behind the ora serrata followed by removal of the lens. Eye is placed in a Petri dish filled with cold PB (with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ). Hold eye in place with forceps and puncture eye with a number 11 scalpel blade just behind the ora serrata. Roll eye onto optic nerve. Use microscissors to cut around the equator. It may be easier to rotate the eye with forceps as you cut. Gently peel the front portion of the eye away from the eyecup. Roll the lens out of the way. Tilt the eyecup on its side to drain the remaining vitreous. *Note:* Any remaining vitreous will crystallize when frozen and create problems for sectioning. The vitreous freezes at a different rate causing the nerve fiber layer and the ganglion cell layer to be pulled away from the remaining retina. Also the remaining vitreous will fracture during sectioning. This causes the block to cut unevenly. The section will buckle.
4. Eyecups are transferred (via a cut plastic transfer pipette) to the fixative solution. Tissue is fixed for 5–20 min by immersion in 4% PFA. We use glass vials for fixation to avoid plastics that may leach from other containers. PB is used because PBS has a higher osmolarity that can cause tissue damage and poor tissue preservation.
5. The fixed eyecups are washed in PB and then cryoprotected at 4°C by sequential immersion in ice cold 10, 20, and 30% (w/v) sucrose in PB. Use a cut plastic transfer pipette to gently move eyecups into each new solution. Allow each eyecup to sink to the bottom of the vial at each step. (About

10–30 min.) The eyecups can sit overnight in the 30% sucrose at 4°C. The eyecups are then immersed into OCT (Sakura Finetek, Torrance, CA). Gently mix to remove the extra 30% sucrose. You should be able to see the mixing of the sucrose and the OCT. If extra sucrose remains inside the eyecup this will also cause sectioning problems. The sucrose will crumble during sectioning and the retina will be pulled along with the sucrose.

6. The tissue is then embedded in OCT (use plastic molds, such as the cap of a microcentrifuge tube, to help position the eyecups) and quickly frozen by immersion into isopentane/dry ice. Isopentane is placed in a glass beaker. Dry ice is packed around the beaker. Allow for the isopentane to equilibrate (otherwise freeze fracture will occur). The tissue is frozen and equilibrated when bubbles no longer form. The OCT-embedded eyecups can be stored at –80°C, but it is preferable to proceed to the sectioning.
7. The eyecup is removed from the molds and cut at 12–15 µm on a cryostat at –18 to –21°C (2 h equilibration in the cryostat is best). Optimal cutting temperature is determined by the humidity in the cryostat chamber and section thickness. Sections are collected onto Super-Frost glass slides, air-dried (this helps keep the sections attached to the slide during immunostaining and gently dehydrates the tissue for best morphology), and stored at –80°C until used for staining.

### 3.1.2. Immunostaining of Retinal Sections

1. Eyecup sections are thawed to room temperature (RT) and dried. Create a circle around the section with a PAP pen and allow to dry. Block by incubation at RT for 30–60 min in AIS.
2. Aspirate off solutions to preserve the well created by the PAP pen. Incubate with primary antibody diluted in AIS for either 1–2 h at RT or at 4°C overnight. (Use a humid chamber to prevent the sections from drying. Use only enough antibody solution to cover the section. Otherwise the solution will flow over the well.)
3. After three quick washes (5–10 min each) in PBS, the sections are incubated for 1 h at RT in the appropriate secondary antibody coupled to either Cy3 or Cy2 (Jackson ImmunoResearch Laboratories, West Grove, PA), or to Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen, Carlsbad, CA) diluted 1:500 to 1:2000 in PBS or AIS. (Keep sections protected from light beyond this step.)
4. The slides are washed again three times in PBS (5–10 min each) with one rinse with water (to reduce salt crys-

tal formation) and then coverslipped with AMM. The coverslipped slides are left in the dark overnight to harden before oil immersion lenses are used. (Nail polish is used to seal the edges of the coverslip.)

5. Slides are viewed on a fluorescence or confocal laser scanning microscope using 40× or 60× oil immersion objectives.

### 3.1.3. Wholemount Retina Staining

1. Following dissection of a mouse retina, four radial cuts are made to flatten the tissue.
2. The flattened retina is placed on a piece of filter paper to prevent the retina from folding or rolling up.
3. The retina on the filter paper is fixed for 1–30 min by immersion in 4% PFA, and then washed in PBS.
4. The tissue is gently removed from the filter paper (this becomes easy after fixation) with a fine paintbrush and is then incubated with primary antibody diluted in AIS for 1–3 days at 4°C on a gently rocking (or orbital) platform.
5. After three 1-h washes in cold PBS, the tissue is incubated overnight at 4°C in the appropriate secondary antibody coupled to either Cy3 or Cy2 (Jackson ImmunoResearch Laboratories, West Grove, PA), or to Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen, Carlsbad, CA) diluted 1:500 to 1:2000 in PBS, again on a rocking platform. (Keep dark.)
6. The tissue is washed again three times for 1 h each in PBS and then carefully placed on a slide, photoreceptor side up. Using a fine paintbrush, the retina is gently flattened and unfolded and then coverslipped with AMM.
7. The coverslipped slides are left in the dark overnight to harden before oil immersion lenses are used. The edges of the coverslip are sealed with nail polish.
8. Slides are viewed on a confocal laser scanning microscope (such as the Olympus FluoView 1000) using 40× or 60× oil immersion objectives.

### 3.2. Electroretinogram (ERG) Recordings

We record the ERG from genetically altered mice to investigate the roles of different proteins in retinal signaling. Below we detail our particular methods for recording the ERG from the mouse. The associated *Notes* provide more in-depth discussion of methods that vary between laboratories. For a detailed description of ERG components and their cellular origin see Frishman (3). Peachey and Ball (4) and Weymouth and Vingrys (2) provide excellent overviews of the recording, analysis, and interpretation of rodent ERGs

1. Prior to the day of ERG recording, make an anesthetic cocktail by mixing 0.5 ml ketamine (100 mg/ml), 0.25 ml



- xylazine (20 mg/ml), and 4.25 ml saline solution (0.9% NaCl for injection; Hospira, Inc., Lake Forest, IL) in a 10-ml sterile mix bottle.
2. Weigh mice and then leave them in the dark to adapt overnight (>12 h).
  3. On the day of ERG recording, mice are anesthetized and prepared for recording under dim red light.
  4. Anesthetize mice via intraperitoneal injection of anesthetic cocktail (*see* **Notes 4–6**). Dose (ml) = weight (g)  $\times$  0.01 (e.g., for a 20-g mouse: dose = 20  $\times$  0.01 = 0.2 ml). Larger doses should be split between two injection sites for better results.
  5. Anesthesia is maintained by subsequent injection of a one-third dose (0.07 ml for 20 g mouse) at 30 and 60 min after initial loading dose. For long procedures we taper anesthesia down to 1/4 dose (0.05 ml for 20 g mouse) for injections at 90 and 120 min after the loading dose.
  6. As soon as the mouse is sufficiently immobilized, it should be placed on the heating device to minimize any drop in core body temperature.
  7. Anesthetize the cornea with a drop of 1.0% proparacaine. After a minute, remove excess fluid with a cotton-tipped applicator (“Q-tip”) as excess fluid can reduce ERG amplitude. Mice can aspirate on drops so care should be taken to ensure excess fluid does not roll down snout.
  8. Dilate the pupils with a drop of phenylephrine (2.5%) and a drop of tropicamide (1%) and again remove excess fluid.
  9. Place a wire loop behind the upper teeth and draw mouse into the nose cone. Start oxygen and fix the wire loop in place so that the head cannot move. (*See* **Fig. 2.2, top**, and **Note 7**.)
  10. Insert rectal temperature probe.
  11. Insert platinum subdermal needle electrode into tail to serve as ground.
  12. Insert butterfly needle into flank to allow delivery of anesthetic every 30 min or as required.
  13. Tape down temperature probe, subdermal needle, and butterfly needle to heating unit to ensure they do not move during recording.
  14. A blanket placed over the mouse will help maintain body temperature.
  15. Attach reference and active electrodes (*see* **Fig. 2.2, top**, and **Notes 8–10**).

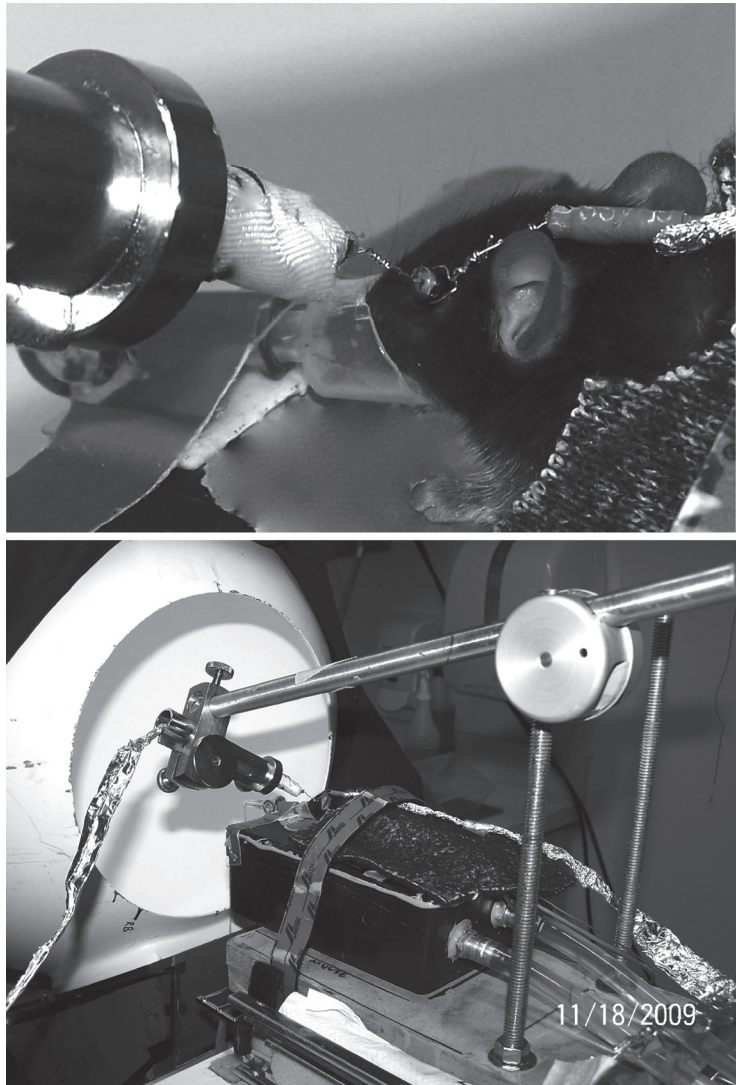


Fig. 2.2. Mouse ERG recording. *Top*: Loop electrode placed over the eye serves as reference. Contact lens electrode placed against the cornea serves as the active electrode. Mouse is stabilized by drawing snout into a nose cone that also delivers oxygen. *Bottom*: Overview of station that holds mouse during recording. Mouse placed on water-circulated heating box. Active corneal electrode held in place by a stand with manipulator at far end. Foil placed over electrode wires is input grounded on the amplifier to reduce 60 Hz interference. After setup is completed, mouse is slid into Ganzfeld dome for ERG recording.

16. To minimize 60 Hz interference, connect the foil shields covering each electrode wire to the amplifier input ground.
17. Slide the mouse forward so that its head is inside the Ganzfeld.

18. Record ERG to a single dim flash (e.g.,  $-3 \log \text{sc cd s/m}^2$ ) to ensure a good contact has been made.
19. Dark adapt the mouse for a further 10 min before starting to record the ERG (see ERG protocol below).
20. At the end of recording, wash the electrodes and contact lens in warm soapy water to remove old Murocel and any proteins, rinse with alcohol. Cleaning will extend the life of the electrodes.

### 3.2.1. Recording Conditions

**Table 2.1** lists our standard protocol for ERG recordings from the mouse. Total recording time including setup is typically 1.5 h. This protocol is quite extensive and based on our requirements to examine retinal signaling. The full protocol may not be necessary depending on your requirements. For example, screening for retinal degenerations could be done using just the “b wave” and “photopic” parts of the protocol.

**Table 2.1**  
**Standard ERG protocol**

ERG component	Flash intensity range (log cd s/m <sup>2</sup> ) <sup>a</sup>	Number of responses averaged	Flash separation (s)	High pass filter (Hz)	Gain
STR	−6.8	60	2	30	10,000
	−4.3	5	3		
b wave	−4.9	10	3	300	2,000
	−1.9	2	10		
a wave	−0.8	1	30	1,000	2,000
	2.1	1	180		
Photopic <sup>b</sup>	−0.8	40	1	300	10,000
	3.7	2	10		

For all ERG recordings: low pass filter = 0.1 Hz, sampling rate = 2.5 kHz, no notch filter.

<sup>a</sup>Each box contains two numbers covering the range of values used for each condition.

<sup>b</sup>Photopic ERGs are recorded 20 min after the onset of a 60-cd/m<sup>2</sup> achromatic background.

Signal averaging is the only way to obtain a useable ERG at low light levels. For example, at the lowest light intensity, we average 60 responses recorded every 2 s in order to obtain a measurement of the scotopic threshold response (STR). Signal averaging increases the signal-to-noise ratio by utilizing the principle that the retinal signal will always be time-locked to the light flash, while non-retinal signals will be random. Therefore, when multiple responses are added, the retinal signal will always superimpose while random signals will tend to cancel each other out. By this

process, the signal-to-noise ratio increases proportionally with the square root of the number of responses averaged. For example, increasing the number of responses averaged from 4 to 16 will double the signal-to-noise ratio.

### 3.2.2. ERG Recording: Useful Tips and Troubleshooting

1. *Main line interference* (50 or 60 Hz) is generated by any equipment using a main line voltage, and this signal is picked up by the electrode wires. Sixty Hertz interference can be minimized either by placing the mouse and recording electrodes in a Faraday cage, or by using the notch filter on the amplifier to completely remove all 60 Hz signal from the ERG. We do not recommend using a notch filter to remove 60 Hz, since we have found significant ERG signal at this frequency in both the ERG *a wave* and oscillatory potentials. Instead, we use a twofold approach in overcoming unwanted 60 Hz contributions to the ERG. The first approach involves running the electrode wire through a grounded shield. We wrap the electrode wires in aluminum foil, which is then connected to the input ground. Using this simple method, we reduce the peak-to-peak amplitude of 60 Hz interference to  $<10\ \mu\text{V}$ . During recording, main line interference greater than  $10\ \mu\text{V}$  is typically caused by either incorrect grounding of the electrode shields, or from poor contact between the active electrode and the cornea. This latter problem can usually be solved by the addition of a small drop of Murocel to the active electrode.
2. *Offset signal averaging* is the second approach we use to minimize 60 Hz interference from ERG recordings. The principle involved is illustrated in **Fig. 2.3a** and **b**. If the time between flashes (inter-flash interval; IFI) is set to 1 s, then the flash will always occur at exactly the same point in the 60 Hz cycle. This is because the main line frequency of 60 Hz is not random and by definition there are exactly 60 cycles in 1 s. Offset sampling involves adding a small-time increment to the 1 s IFI such that each flash will occur at a different point in the 60 Hz cycle. **Figure 2.3a** shows five different starting points in a 60-Hz cycle. The first flash will start sampling from point 1, the second flash will start sampling from point 2, etc. **Figure 2.3b** shows the first 20 ms of each 60 Hz waveform from each starting point in **Fig. 2.3a**. The average of these five waveforms is zero (**Fig. 2.3b**, horizontal line).

The offset signal averaging method demonstrated in **Fig. 2.3a** and **b** was achieved by setting the inter-flash interval to 1.00333 s. The addition of the 3.33 ms shifts the start of sampling by  $1/5$  of the 60 Hz cycle (i.e.,  $1/5 \times 16.667\ \text{ms}$ ). Averaging any number of flashes that is a

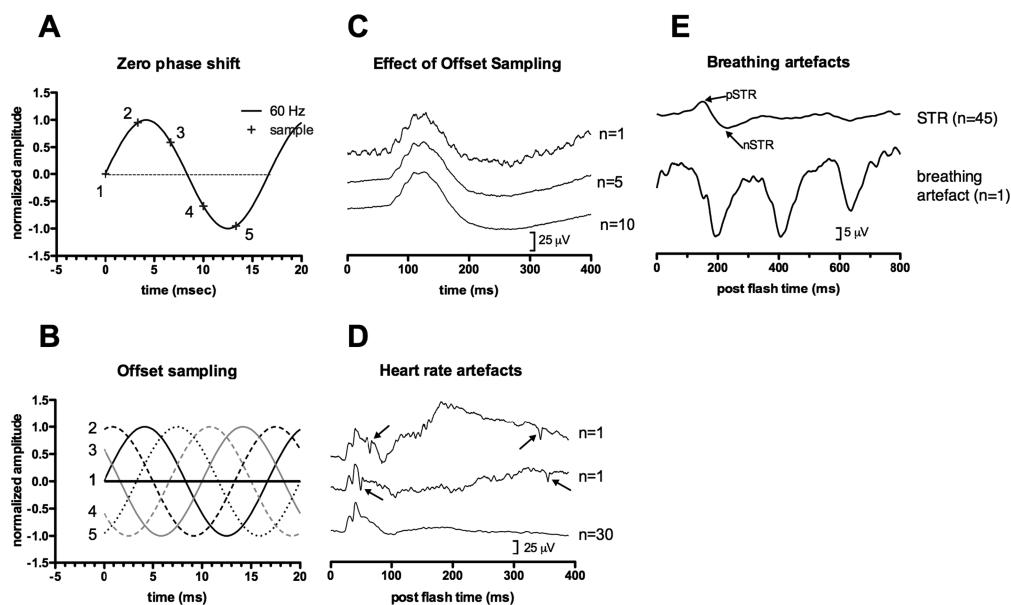


Fig. 2.3. Minimizing 60 Hz, breathing and heart rate artifacts in the mouse ERG. **a** Solid line shows 1 cycle of a 60-Hz waveform which lasts 16.667 ms. The number points show starting locations of sampling in five sequential epochs. **b** Each trace shows the time course of the 60 Hz cycle for the 20 ms following each starting point. The horizontal line shows the average of the five waveforms. **c** Scotopic ERG response to a dim flash ( $-3.2 \log \text{sc cd s/m}^2$ ) in response to a single flash (top) or for the average of 5 or 10 responses. **d** Photopic ERG response to a flash ( $1.2 \log \text{ph cd s/m}^2$ ) presented against a achromatic background ( $60 \text{ cd/m}^2$ ). Top two traces show responses to a single flash and arrows show intrusion of the heart beat into the ERG response. Bottom trace shows the average of 30 responses and the heart beat, which is not synchronous with the flash, can no longer be seen. **e** The STR obtained from the average of 45 ERG responses recorded to a very dim flash ( $-6.1 \log \text{sc cd s/m}^2$ ). Note that the peak-to-peak amplitude is only about  $10 \mu\text{V}$ . Below the average is a single response over the 80 ms following this flash. The large oscillations are breathing artifacts in which the body movements of the mouse are causing the eye to move relative to the active electrode and are thus recorded.

multiple of 5 will result in a zeroing of the main line 60 Hz interference (see Note 11). Figure 2.3c shows the effectiveness of offset signal averaging. The ERG response to a single dim flash (top trace) has prominent 60 Hz interference. The bottom two traces show that this non-physiological effect is no longer present after offset signal averaging of 5 or 10 traces. Importantly, any physiological 60 Hz component of the ERG will not be eliminated since this signal will always be synchronous to the flash.

3. *Heartbeat artifacts*: It is not uncommon for the heart beat to appear on ERG traces (Fig. 2.3d; top two traces). However, since the heart beat is not synchronous with the flash, evidence of the heart beat is undetectable in the averaged response (Fig. 2.3d, bottom trace). If the heartbeat artifact is large enough to be a problem, moving the electrode position may be required, although this is rarely necessary.

4. *Breathing artifacts*: The *bottom trace* in **Fig. 2.3e** shows an example of a breathing artifact, which is commonly observed in mouse ERG responses. The breathing artifact is caused by excessive body movement, which in turn moves the eye back and forth across the corneal electrode. We have found that breathing artifacts are minimized with supplemental oxygen and by stabilizing the head. In order to stabilize the head, we place a wire loop behind the upper teeth and draw the mouse into a nose cone, which also delivers oxygen (**Fig. 2.2a**). After being drawn into the nose cone, the wire loop is tied off such that the head is held firmly in place. A large breathing artifact may also be seen when anesthesia is too light. As for the 60 Hz and heart rate artifacts, sufficient averaging will zero the breathing artifact and enable the ERG signal to be obtained. The *top trace* in **Fig. 2.1e** shows the STR obtained for an extremely dim flash by averaging 45 responses each of which had the breathing artifact seen in the bottom response.
5. *Cataracts*: Mice develop cataracts quite soon after injection with the anesthetic cocktail used for ERG recording. However, these cataracts do not pose a problem to ERG recording since they act as a natural light diffuser and very large ERG amplitudes are still obtained even with apparently dense cataracts. Cataract formation is minimized by keeping the animal warm and the cornea lubricated.
6. *Small ERG amplitudes*: Smaller than expected ERG amplitudes can be attributed to poor positioning of the electrode against the cornea (i.e., electrode is not centered), poor electrode contact with the cornea, or poor positioning of the inactive electrode. For example, with our electrode configuration, smaller amplitudes are obtained if the reference loop electrode is not placed fully behind the eye. Additionally, adding too much Murocel to the contact lens will also reduce ERG amplitude as it shorts the electrical contact between active and loop electrodes.

### 3.2.3. ERG Notes

1. We use a custom-made recording system based on ERGTool software (kindly supplied by Dr. Richard Weleber, Casey Eye Institute, Portland, OR), installed on a G4 Macintosh Computer running OS9. The system also requires a custom-made interface box and National Instrument 16 channel A/D boards. A new version for the Intel chip-based Macintosh Computers running OSX is currently under development. We use a number of light sources mounted together in one box and channeled to the opening on a 40-cm Ganzfeld via a light tunnel. High-intensity flash stimulation is provided by photoflash units



(2405CX and a modified 1205CX power supplies with 205 flash units: Speedotron, Chicago, IL). Low-intensity flash stimulation is provided by a Grass PS22 (Astro-Med, West Warwick, RI). Flash intensity is adjusted by the use of metallic neutral density filters, and the wavelength is modified with glass color filters (Melles Griot, Optics Group, Rochester, NY).

2. The use of xylazine as an anesthetic has the advantage that it prevents the eye moving. Two unwanted side effects include suppressed respiration and a dramatic reduction in core body temperature. We supply a low flow of oxygen through a nose cone, and the mouse is placed on a water-circulated heating source during recording (**Fig. 2.2**).
3. We built a water-circulated heater using a 3.5" × 6" electronics box from Radioshack. A cutout, slightly larger than the size of a mouse, was made in the plastic top of the box. The metal plate that comes with this electronics box was then glued to the underside of the plastic top using marine grade epoxy available from hardware stores. Two 1/2-inch copper connectors, which carry the heated water, were glued into one end of the box. Finally the modified plastic/metal top was glued to the box using the marine grade epoxy. Water is circulated through the box via 1/2-inch plastic tubing that carries the water from a laboratory water heater (**Fig. 2.2**).
4. Anesthetic dose (ml) = [weight (mg) × dose (mg/g)]/concentration (mg/ml). For a mouse we use a ketamine dose of 0.1 mg/g. The ketamine concentration of the cocktail = 10 mg/ml. Therefore, for a 20-g mouse:
  - a. Dose (ml) = [20 g × 0.1 mg/g]/10 mg/ml = 0.2 ml.
  - b. The cocktail is mixed at ketamine to xylazine ratio of 10:1; therefore, the loading dose for xylazine is 0.01 mg/g.

An anesthetic protocol is not something that can be set in stone. Even in animals that are from the same litter, anesthetic requirements can differ greatly between animals depending on many factors such as fat content and excitability. Mice with a lot of adipose tissue or that are highly agitated/excited at initial injection may need more initial anesthetic to reach a suitable anesthetic plane for ERG recording. For some mutant mice, we lower the anesthetic dose because of increased mortality during ERG recording. Alternatively, in one strain, we had to increase the anesthetic dose in order to achieve 30 min of sedation between re-doses.

5. Eye movements and the presence of breathing artifacts on ERG traces and/or direct visualization of whisker movements are all used to assess the depth of anesthesia during testing. As the mouse passes from the alert state to the required anesthetic plane, whisker movements slow down and eventually stop and the breathing artifact disappears from the ERG response. Conversely, during ERG recording, the appearances of eye movements, breathing artifacts, or whisker movements indicate that a re-dose of anesthetic is required.
6. Anesthetic protocols vary between laboratories, each of which finds a protocol that works for them. Most laboratories use a ketamine:xylazine (K:X) loading dose followed by a re-dose at varying time intervals, e.g., Hetling and Pepperberg (5) (K:X = 0.15:0.01 mg/g; re-dose 1/8 to 1/4 at 45 min); Saszik et al. (6) (K:X = 0.07:0.007 mg/g; re-dose K:X = 0.072:0.005 mg/g at 45 min); and Peachey (4) (K:X = 0.08:0.016 mg/g; given as 75% initial dose then remaining 25% 10 min later, re-dose K:X = 20% of initial dose after 30–40 min). Woodward (7) used isoflurane for mouse ERG recordings as they found an unacceptably high mortality rate with ketamine and xylazine in their mouse mutants.
7. Oxygen delivery and head stabilization help minimize breathing artifacts and lower anesthetic deaths. We fashioned a nose cone from a cutoff 3 ml syringe: The syringe is pushed into one end of a 3-way valve. A cutoff 3/16-inch nasal canula is inserted into the “T” of the valve and carries oxygen from the tank. A suture bent into a hook is placed under the upper teeth of the mouse and serves to pull the mouse into the nose cone and hold the head steady. During setup, the suture line is fed through the syringe and 3-way valve. Once the mouse is pulled firmly into nose cone, the suture line is held tight by wrapping it around a small cleat attached to the front of the heating box.
8. We use a custom-made contact lens electrode placed on the cornea for the active electrode and use a loop placed over the proptosed eye as the reference (**Fig. 2.2**). The contact lens is formed from heated Aclar plastic. A hole is pierced through the center of the contact lens with a heated 28-ga needle. Platinum electrode wire, with one end formed into an “L” shape, is threaded through the center of the contact lens. The contact lens can move freely along the electrode wire. For ERG recording, the L-shaped end of the electrode wire is pushed up against the cornea. The contact lens with a very small amount of Murocel is then moved down the wire with a pair of fine forceps until the



lens covers the cornea. The contact lens helps prevent drying of the Murocel, thereby producing a more stable ERG for a longer period.

9. The reference loop electrode is placed over the proptosed eye. Once over the eye, the loop is pulled back gently to ensure the loop is in good contact with the front of the eye (**Fig. 2.2, top**). The electrode wire is run over the head between the ears and held in position with Velcro straps (**Fig. 2.2, bottom**). Both active and reference electrodes are made from approximately 2–3-inch long sections of platinum wire (0.01 inch diameter; World Precision Instruments, Sarasota, FL). One end of each electrode wire is wrapped tightly around blunted platinum subdermal needle electrode wires (Astro-Med Grass, West Warwick, RI).
10. Electrode configurations vary widely across laboratories. An L-shaped wire placed against cornea is commonly used for the active electrode. However, the material used varies, with some preferring Ag/AgCl, stainless steel, or precious metals such as gold or platinum. Another option is a DTL fiber (cotton fiber-embedded Ag/AgCl particles) held in place with a contact lens. The advantage of Ag/AgCl electrodes is that they produce a very stable response with little drift and, therefore, the high pass filter can be set for direct current recording (i.e., 0 Hz cutoff). The disadvantage of Ag/AgCl electrodes is that they produce very large flash artifacts in response to bright flashes. We use platinum for all our electrodes since it provides a stable ERG response with no flash artifact. For our ERG recording we set the high pass filter to 0.1 Hz. The choice of reference location varies considerably between laboratories. Reference electrodes used include a DTL fiber under an opaque contact lens on the eye contralateral to the one tested, a subdermal needle electrode inserted in the cheek beneath the eye tested, and metal electrodes placed in the mouth. The ERG is a measure of the change in voltage across the retina in response to a light stimulus. For this reason, we use the loop placed over the eye as reference because it enables electrodes to be essentially placed on either side of the retina and allows the most direct in vivo method of measuring the voltage across the retina.
11. Our system allows microsecond control of timing. In other systems, offset averaging can still be used even if there is less control of the time between flashes. For example using an offset of 2 ms and averaging multiples of 8 responses (total = 16 ms instead of 16.667 ms) will still reduce 60 Hz interference.

### **3.3. Measuring Vision in Rodents**

Laboratory rodents may seem like a peculiar choice as animal models for studying the human nervous system, particularly ones for testing visual function because rodents have relatively small eyes, have a small visual cortex, and have a predominately rod-based retina. However, upon closer comparison, the rodent and primate visual systems are quite similar and many of the same visual functions can be measured in both species. Although, given the architecture of the human macula and the comparable size difference between human and rodent brains, the human visual system outperforms that of the rodent. For example, average human visual acuity is approximately 30 cycles/degree (cpd) (20/20), whereas the average visual acuities of rats and mice are  $\sim 1.0$  cpd (20/600) and 0.6 cpd (20/1000), respectively. Although visual acuity is lower in rodents compared to humans, rodents do have quantifiable vision, which can be quite detailed at close distances (8). Furthermore, it has recently been shown that complex visual capabilities such as motion coherences, which were thought to not exist in rodents, are not only quantifiable, but in fact, rodents are quite good at them (9).

Since the 1930s, a number of methods have been devised to quantify rodent vision. Lashley's jumping stand may have been the first method used to quantify rat vision (10, 11), and it is still used to a limited extent. Y-mazes (11), conditioned aversion (12), and operant tasks (13, 14) have all been employed with some success, but in general, these methods require a considerable amount of time to train and test rats, which probably accounts for their limited popularity. In addition, the harsh negative consequences of an incorrect response, in particular the ones used with Lashley's jumping stand, are not optimal in training and testing animals behaviorally. Some experimenters have also used a modification of the Morris water task in which rats learn to swim to a platform that is raised above the water's surface, guiding themselves with the use of visual cues (15). However, viewing distances are hard to control in this situation, making quantitative measurements nearly impossible. Since then, two methods have emerged that are designed to minimize the constraints and limitations of previous methods and allow for quantitative measurements.

#### **3.3.1. The Visual Water Task**

The Visual Water Task (VWT; **Fig. 2.4**) is an apparatus used to evaluate visual perception of rodents in a psychophysical manner. Animals are trained to discriminate between two visual stimuli and are positively reinforced for each correct response. The apparatus consists of a trapezoidal-shaped tank containing water, with two computer monitors facing through a clear glass wall into the wide end of the pool. Visual stimuli are generated and projected on the screens using a computer program (Vista<sup>®</sup>; CerebralMechanics). The choice point, defined by a 46-cm long midline divider,

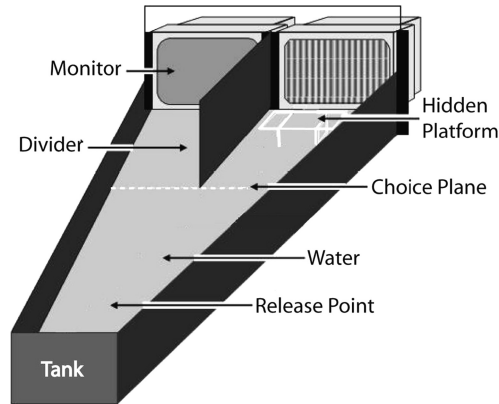


Fig. 2.4. The Visual Water Task. A task used to evaluate the visual perception of rodents (8, 9).

which extends into the pool from between the monitors, creates a Y-maze with a stem and two arms. A moveable, transparent Plexiglas escape platform (37 cm L  $\times$  13 cm W  $\times$  14 cm H) is always submerged directly below whichever monitor displays the grating. A LRLRLRR sequence, a pattern the animals cannot memorize, is used for the location of the gratings. Animals are released into the pool from the wall opposite the monitors, and the end of the divider within the pool sets a choice point for the rats that is as close as they can get to the visual stimuli without entering one of the two arms. The length of the divider, therefore, sets the effective spatial frequency of the visual stimuli. The animals usually stop at the end of the barrier and inspect both screens before choosing a side. If the animals swim to the platform below the positive stimulus without entering the arm with the monitor showing the negative stimulus, the trial is considered correct; if they swim into the arm of the maze that contains the negative stimulus, the trial is recorded as an error.

The most common measure of vision is acuity, measured clinically with the use of a Snellen chart. This technique measures the ability to resolve two high contrast items as distinct; two parts of the same letter on a Snellen chart. Discrimination between sine wave gratings and a gray screen of the same mean luminance in the VWT is akin to Snellen acuity and allows researchers to measure visual acuity in rodents in a similar manner to those measured clinically, helping validate experimental results. Animals are first trained to discriminate between a low spatial frequency ( $\sim 0.1$  cpd), vertical sine wave grating (+ stimulus; 100% contrast), and uniform gray of the same mean luminance (36.2 cd/m<sup>2</sup> at the choice point). The animals are tested in groups of 5 or 6, with 15–20 interleaved trials each, with each session lasting 45–60 min. No more than two sessions, separated by at least 1 h, are performed in

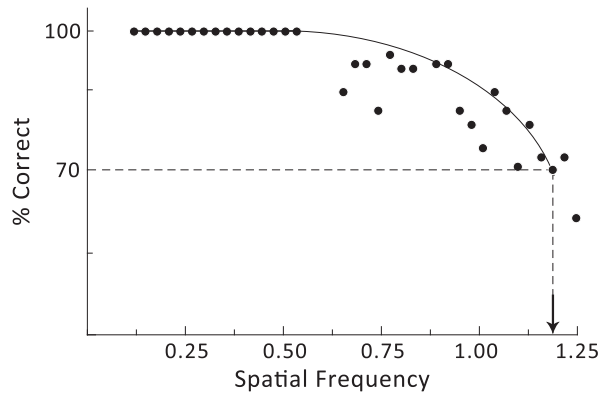


Fig. 2.5. An example of a frequency-of-seeing curve. Frequency-of-seeing curves are used to estimate the visual acuity based upon a 70% correct criterion.

a single day. All trials are run with the room lights off. Once animals achieve near-perfect performance (90% or better over at least 40 consecutive trials), the animals are then tested for visual acuity. A flexible method-of-limits procedure is used in which incremental changes in the spatial frequency of the sine wave grating are made until choice accuracy falls below 70% (**Fig. 2.5**). Accuracy for a given frequency is measured in blocks of ten trials when near threshold, and shorter blocks at the low spatial frequencies, thereby minimizing the number of trials far away from threshold. A preliminary grating threshold is established when animals fail to achieve 70% accuracy at a spatial frequency. In order to assess the validity of this estimate, the spatial frequency of the grating is increased by about 0.1 cpd, and the experimental procedures described above are repeated until a stable pattern of performance is established. The performance at each spatial frequency is averaged for each animal and a frequency-of-seeing curve is constructed. The point at which the curve intersects 70% accuracy is recorded as the visual acuity (**Fig. 2.5**).

Contrast sensitivity is assessed using similar procedures, except the minimal contrast required to differentiate between the screens at different spatial frequencies is measured (**Fig. 2.6**). Contrast thresholds are typically measured after grating acuity is assessed, so minimal re-training is required. Seven spatial frequencies are normally tested; 0.059, 0.119, 0.208, 0.297, 0.505, 0.712, and 0.890 cpd. At each spatial frequency, trials are initiated at 100% contrast and the contrast is decreased systematically until performance falls below 70% accuracy. The contrast threshold is measured independently at least three times, after which final values are computed as above from frequency-of-seeing curves of the combined data. Measuring responses to different types of visual stimuli can test a range of visual functions, beyond visual acuity and contrast sensitivity. These include oblique gratings, color gratings, moving gratings, and dot motion among

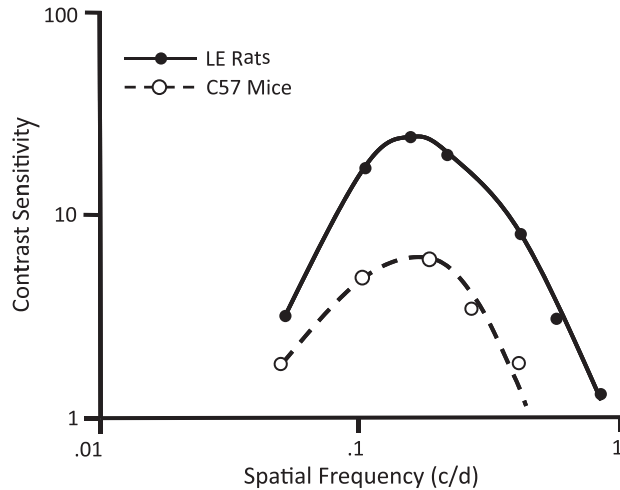


Fig. 2.6. Contrast sensitivity curves generated using the Visual Water Task.

others (8, 9, 16). Testing each of these visual functions follows the same general procedure, only the visual stimuli are systematically changed.

A significant limitation of the VWT is that the time invested in training and testing animals is on the order of weeks, which limits longitudinal studies to a maximum measurement frequency of once per month. On the other hand, once the animals are well trained, they do not require a full re-training before subsequent testing, but rather, a day or two of swimming trials are sufficient to restore optimal performance. Visual thresholds are generated by compiling data collected over the course of 1–2 weeks; therefore, daily measurements of vision are not possible in the VWT. As a consequence, the earliest age at which visual thresholds can be measured from rodents in the VWT is approximately P30, about 2 weeks after the day of eye opening.

### 3.3.2. The Virtual Optomotor System

The Virtual Optomotor System (**Fig. 2.7**) is primarily used to evaluate spatial frequency thresholds (optomotor acuity) and contrast sensitivity. The apparatus consists of four computer monitors positioned around a square testing arena. An unrestrained rat is placed on a platform in the center of the arena, and a sine wave grating drawn on a virtual cylinder is projected on the monitors in 3D coordinate space (OptoMotry®; CerebralMechanics). A video camera provides real-time video feedback from above, and the position of the head on each frame is used to continually center the hub of the cylinder at the rat's viewing position. On each trial the cylinder is rotated at a constant speed ( $12^\circ/\text{s}$ ) and the experimenter judges whether the rat makes tracking movements with its head and neck to follow the drifting grating. The spatial frequency threshold, the point at which animals are no longer able to

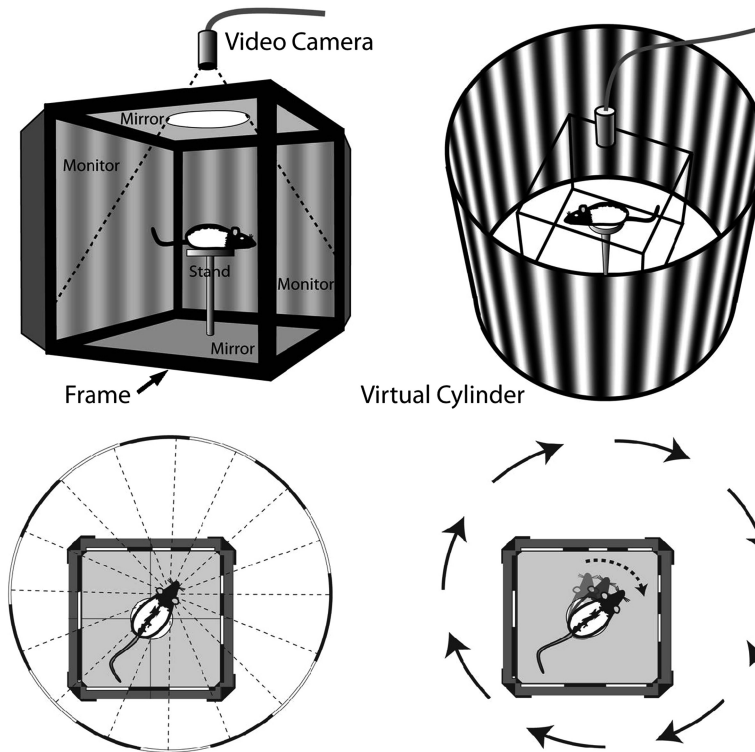


Fig. 2.7. The Virtual Optomotor System (VOS). *Top left*: side view of the apparatus, which consists of four computer monitors facing inward (two removed for ease of viewing) into an arena. An animal is placed on a platform located in the middle of the arena and a video camera viewing from above provides real-time feedback of animals' behavior. *Top right*: the virtual cylinder displaying sine wave gratings with the testing arena and animals highlighted within. *Bottom left*: diagram depicts the animals viewing angle of the visual stimuli. The sine wave gratings are displayed as different sizes on the monitors in order to keep the virtual cylinder homogeneous throughout the testing area. *Bottom right*: arrows show the direction of rotation of the virtual cylinder and the corresponding movement of an animal performing the optokinetic tracking behavior (17, 21).

track, is obtained by incrementally increasing the spatial frequency of the grating at 100% contrast. Contrast sensitivity thresholds are measured at up to eight different spatial frequencies by systematically decreasing the contrast until no tracking is observed. Thresholds through each eye are measured separately by simply reversing the rotation of the cylinder (17).

One of the major advantages for using the VOS is that animals require no reinforcement training prior to being tested. This greatly decreases the time required to generate a visual threshold, and therefore allows for much larger group sizes to be examined. In addition, the ability to generate thresholds quickly, in some cases as quickly as 5–10 min, allows for thresholds to be assessed on a daily basis. This is of particular importance in developmental studies as animals can be tested from the day of eye opening and for very fast retinal degenerations where visual decline can occur sooner than it can be measured with other tasks. Another

advantage arises from a combination of inherent properties of the task itself and the structure of the rodent visual system. That is, when the virtual cylinder that displays the sine wave grating is rotated in the clockwise direction, only the left eye (tracking in the temporal to nasal direction) responds to the movement, resulting in an elicited optomotor behavior moving in the same direction as the grating movement. Conversely, if the cylinder is rotated in the counter-clockwise direction, only the right eye (again temporal to nasal) responds to the stimuli. This phenomenon is particularly advantageous as therapies for retinal disease are often performed monocularly, maintaining the contralateral eye as a control. Therefore, employing the VOS allows for within animal controls to be used in the experimental design, a powerful method in research. Finally, the VOS is also designed to provide a “blind” psychophysical testing methodology, which can be used to eliminate operator bias. This is of particular importance when evaluating the efficacy of an experimental therapy.

### *3.3.3. Differences Between the Virtual Optomotor System and the Visual Water Task*

There are many different aspects of vision such as visual acuity, visual motion, color vision, and form vision. The VOS and the VWT are tasks designed to evaluate two different forms of visual thresholds, reflexive optomotor responses and perceptual visual acuity, respectively. Because the tasks evaluate different components of vision, the sensory and motor circuits each task relies upon are likely to be different. For example, the VWT is normally dependent on the visual cortex, and surgical removal of the visual cortex (V1) results in significantly lower visual thresholds (unpublished observations). The VOS, however, is normally mediated by subcortical circuits and removal of the cortex does not affect the measured thresholds (16). Finally, the visuospatial thresholds of naïve adult rodents are lower when measured in the VOS than when measured in the VWT, which is likely a reflection of the VOS relying on subcortical circuits and the VWT relying on the visual cortex. Therefore, these and other factors should be taken into consideration in choosing an appropriate method of testing vision.

### *3.3.4. Applicability of the Tasks*

The VWT has been used for a number of studies examining rodent vision. The first published study using the VWT examined the visual acuity of Long Evans rats and C57BL/6 mice (8). This study revealed that behaviorally measured visual acuity in rats is approximately double that of the mouse, although both species could be tested repeatedly and reliably. These studies were then extended into other rodents including albino rats and other pigmented rat strains showing that differences within the same species can also be detected (18). The VWT has also been used to examine different forms of vision such as motion acuity (9), dot motion coherence (9), and acuity using oblique



or horizontal gratings. Since then, the use of the VWT has been extended to include studies involving damage to the visual system. For example, ablation lesions of the visual cortex and visual cortex stroke result in a significant drop in visual acuity from 1 cpd down to  $\sim 0.7$  cpd (unpublished observations). In 2004, McGill et al. (19) performed a longitudinal characterization of visual acuity (measured once every 30 days) of rats that undergo photoreceptor degeneration. The authors showed that from 30 days of age until 11 months, the rats progressively declined from near-normal vision, to the inability to discriminate between a white and a black screen. This was the first longitudinal quantification of spatial vision in a model of retinal disease. This study was followed closely by cell-based therapies used to prevent the degeneration of vision in the RCS rat (20), where both a human-derived RPE cell line (ARPE19) and human Schwann cells were shown to significantly limit the progression of visual deterioration.

The VOS is newer than the VWT and therefore has been used to a lesser extent. However, over the last few years the task has been used to quantify visual thresholds in normal mice (17), developing mice (21), monocularly deprived mice (22), in normal and experimentally enhanced rats (16, 17), and in retinal degenerative rats (23) and rats receiving a potential neuroprotective treatment (24). The VOS has also been used to examine vision in mice without functional rods or cones (25), *noerg-1* (26), and *nob4* mice (27) and TRPM1-deficient mice (28). Finally, the VOS has also been used to evaluate the role of ON bipolar cells that were engineered to be photosensitive (29).

In summary, the Visual Water Task and the Virtual Optomotor System allow for detailed examination and quantification of visual thresholds in both normal and diseased rodent models. These behavioral tasks allow for therapeutic interventions to be evaluated using multiple approaches to testing visual function. Finally, each task measures visual thresholds in a manner similar to those used clinically, helping validate the use of these tasks for vision research in rodents.

Both these tasks and a comprehensive list of scientific publications using these tasks can be found at: [www.cerebralmechanics.com](http://www.cerebralmechanics.com).

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