Video Article

Preparation of Living Isolated Vertebrate Photoreceptor Cells for Fluorescence Imaging

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Abstract

In the vertebrate retina, phototransduction, the conversion of light to an electrical signal, is carried out by the rod and cone photoreceptor cells[1-4]. Rod photoreceptors are responsible for vision in dim light, cones in bright light. Phototransduction takes place in the outer segment of the photoreceptor cell, a specialized compartment that contains a high concentration of visual pigment, the primary light detector. The visual pigment is composed of a chromophore, 11-*cis* retinal, attached to a protein, opsin. A photon absorbed by the visual pigment isomerizes the chromophore from 11-*cis* to all-*trans*. This photoisomerization brings about a conformational change in the visual pigment that initiates a cascade of reactions culminating in a change in membrane potential, and bringing about the transduction of the light stimulus to an electrical signal. The recovery of the cell from light stimulation involves the deactivation of the intermediates activated by light, and the reestablishment of the membrane potential. Ca^{2+} modulates the activity of several of the enzymes involved in phototransduction, and its concentration is reduced upon light stimulation. In this way, Ca^{2+} plays an important role in the recovery of the cell from light stimulation and its adaptation to background light.

Another essential part of the recovery process is the regeneration of the visual pigment that has been destroyed during light-detection by the photoisomerization of its 11-*cis* chromophore to all-*trans*[5-7]. This regeneration begins with the release of all-*trans* retinal by the photoactivated pigment, leaving behind the apo-protein opsin. The released all-*trans* retinal is rapidly reduced in a reaction utilizing NADPH to all- *trans* retinol, and opsin combines with fresh 11-*cis* retinal brought into the outer segment to reform the visual pigment. All-*trans* retinol is then transferred out of the outer segment and into neighboring cells by the specialized carrier Interphotoreceptor Retinoid Binding Protein (IRBP).

Fluorescence imaging of single photoreceptor cells can be used to study their physiology and cell biology. Ca^{2+}-sensitive fluorescent dyes can be used to examine in detail the interplay between outer segment Ca^{2+} changes and response to light[8-12] as well as the role of inner segment Ca^{2+} stores in Ca^{2+} homeostasis[13,14]. Fluorescent dyes can also be used for measuring Mg^{2+} concentration[15], pH, and as tracers of aqueous and membrane compartments[16]. Finally, the intrinsic fluorescence of all-*trans* retinol (vitamin A) can be used to monitor the kinetics of its formation and removal in single photoreceptor cells[17-19].

Video Link

The video component of this article can be found at https://www.jove.com/video/2789/

Protocol

1. Preparation of Sylgard-covered dishes, experimental chambers, and razor blades

1. 35 mm Falcon Petri dishes coated with Sylgard elastomer are needed for the proper chopping of an isolated retina to obtain single photoreceptor cells. The elastomer is prepared according to the supplier's instructions and a small amount is poured into each dish to cover its bottom with a layer. Replace the dish covers after coating and store them. In a few days' time the elastomer hardens and the dishes are ready.
2. Isolated photoreceptors need to stick to the bottom of the experimental chamber so that they are immobilized during the course of an imaging experiment. This is achieved by coating the bottoms of the chambers with poly-L-lysine or poly-L-ornithine. Add 200 µL of 0.01% solution of either one per chamber, and cover the chambers with a paper towel to protect them from dust. After the solution has dried, wash the chambers with distilled water and store in a closed box. Use within 2 weeks.
3. To clean the chambers at the end of an experiment, wash them with 100% ethanol to remove any oil from the oil-immersion lens and cell debris. To remove the cell debris, use cotton-tipped applicators and carefully scrub the bottom of the chamber. Afterwards, wash with distilled water and let the chambers dry before re-coating.
4. Cut double-edged razor blades into small pieces (8 from one blade) with a metal cutter.

2. Preparation of solutions

1. The composition of solutions depends on the species. For amphibians, the Ringer's has (in mmol/L): 110 NaCl, 2.5 KCl, 1.6 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, pH = 7.55. The pH should be adjusted to the final value with NaOH. For mammals, the Ringer's has (in mmol/L): 130 NaCl, 5 KCl, 0.5 $MgCl_2$, 2 $CaCl_2$, 25 hemisodium-HEPES, pH = 7.40. The Ringer's solution can be kept well-sealed at room temperature for a few months.
2. Stock glucose solution, 1 mol/L, kept at -20 °C to avoid bacterial growth.
3. On the day of the experiment, add glucose to the Ringer's solution to a final concentration of 5 mmol/L. At the end of the day, discard the Ringer's that contains glucose because it might grow bacteria.

3. Isolation of retinas

1. For the proper excision of a retina it is important that the animal be dark-adapted for at least 2-3 hours before sacrifice. Animals should be dark-adapted in a suitable ventilated container in the dark-room.
2. Fill two 35 mm Petri dishes half-way with Ringer's solution.
3. Sacrifice the animal under dim red light and remove the eyes. Subsequently, all procedures are carried out under infrared light using a dissecting microscope or a camera with a video monitor.
4. Remove any leftover tissue from the outside surface of the eye. Cut and remove the anterior part, then transfer the eyecup into one of the Petri dishes filled with Ringer's.
5. Remove the vitreous and carefully separate the retina from the rest of the eyecup by pinching off or cutting any attachments. Gently lift the retina and separate it fully from the eyecup.
6. Transfer the retina to the second Petri dish with a plastic transfer pipette. Keep the dish containing the retina in a light-tight box.

4. Isolation of single photoreceptor cells

1. All procedures are carried out under infrared light. Cut a small piece of retina and transfer it with a plastic pipette to a Sylgard-covered dish. The volume of the solution containing the retina piece should be about 250 µL.
2. Grab a small piece of razor blade with the blade holder -- the edge of the blade should be at approximately 45° angle to the holder. Flatten the piece of retina on the Sylgard layer and using the blade chop the piece of retina finely while keeping it stuck to the Sylgard layer.
3. Transfer 200 µL of the solution containing the cells to an experimental chamber – leave any remaining piece of retina in the Sylgard-covered dish. Keep the chamber with the isolated cells in a light-tight box.
4. Wait for 10 min for the cells to settle, then add 2-3 mL of Ringer's. At this stage, the isolated cells can be loaded with a particular fluorescent dye (for example Fura-2) according to the dye loading protocol.
5. The cells can now be taken to the microscope stage for experiments.

5. Fluorescence imaging

1. Transfer the chamber to the stage of the epifluorescence microscope. Adjust solution perfusion, temperature probes, infrared illumination, etc.
2. Close the curtains and begin experiment. Turn on the infrared light inside the microscope cage, focus on the bottom of the experimental chamber, and move the stage looking for cells.

6. Representative Results:

Fig. 1 shows the morphology of healthy isolated rod and cone photoreceptors obtained with this protocol from a salamander (*Ambystoma tigrinum*) retina. Salamander cells have been used extensively for single cell fluorescence imaging studies because of their large size and their ability to survive for several hours after isolation from the retina. In addition, from a salamander retina one can regularly obtain both rod and cone photoreceptors.

One important criterion for the health of the cells is the presence of an intact ellipsoid (**Fig. 1**), the part of the cell where the mitochondria are concentrated. When the cells are viewed under DAPI optics, this concentration of mitochondria gives a strong fluorescence signal (**Fig. 2**) due to the presence of NADH. Lack of an intact ellipsoid is a sign of a damaged cell, generally unfit for experiment. **Fig. 3** shows a damaged salamander rod photoreceptor, with a swollen cell body and a condensed nucleus. Such cells display much lower fluorescence under DAPI optics, but viewed under FITC optics show a strong FAD signal in the ellipsoid region (originating from oxidized flavin nucleotides and flavoproteins). Another criterion for the health of isolated photoreceptors is their ability to generate all-*trans* retinol (vitamin A) in their outer segments upon stimulation by light. The generation of vitamin A requires substantial amounts of NADPH, which depends on an intact metabolic machinery. Figures 4 and 5 show the formation of vitamin A in the outer segments of intact frog and mouse rod photoreceptors respectively.

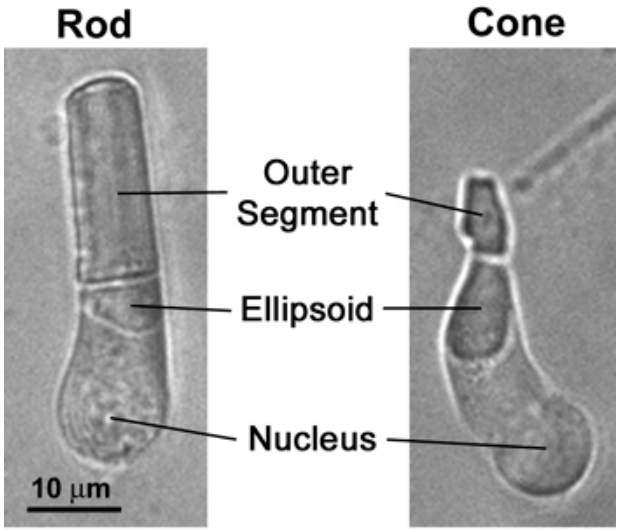

Figure 1. Healthy single rod and cone photoreceptors. The cells were isolated from a tiger salamander retina. Phototransduction takes place in the outer segment and the ellipsoid is densely packed with mitochondria. Rods are responsible for dim light vision, cones for bright light vision.

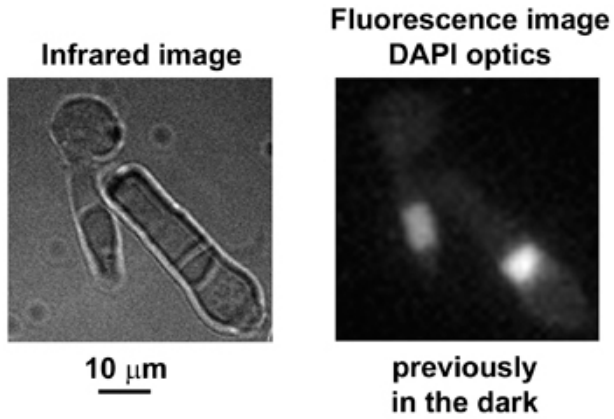

Figure 2. Fluorescence of living salamander rod and cone. These are dark-adapted cells, showing strong NADH fluorescence in their respective ellipsoids and no significant vitamin A fluorescence in their outer segments. The capture of the fluorescence image represents their first exposure to visible light after the period of dark-adaptation.

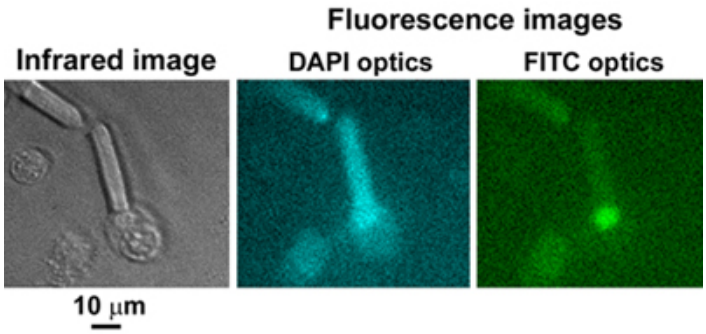

Figure 3. Damaged salamander rod photoreceptor. The swollen cell body and the condensed nucleus are indicative of damage. The cell is oxidized and there is minimal NADH signal (DAPI optics), but much stronger FAD signal (FITC optics).

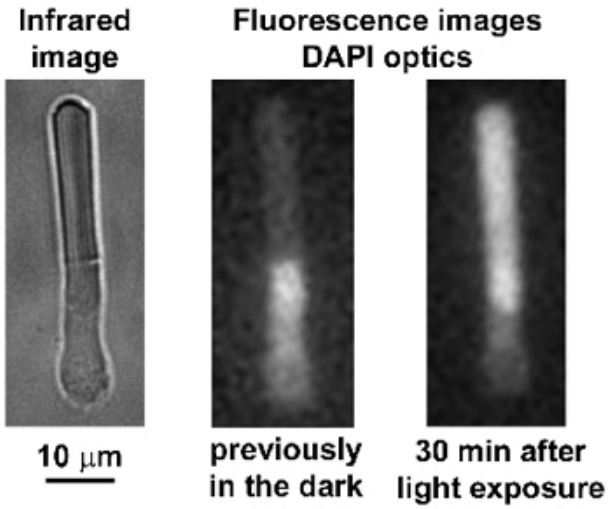

Figure 4. Frog rod with NADH and retinol. This is a healthy frog rod photoreceptor showing strong NADH fluorescence in the ellipsoid region. Before light exposure there is minimal fluorescence in the outer segment. Following light exposure, there is a significant increase in the outer segment fluorescence due to the formation of vitamin A.

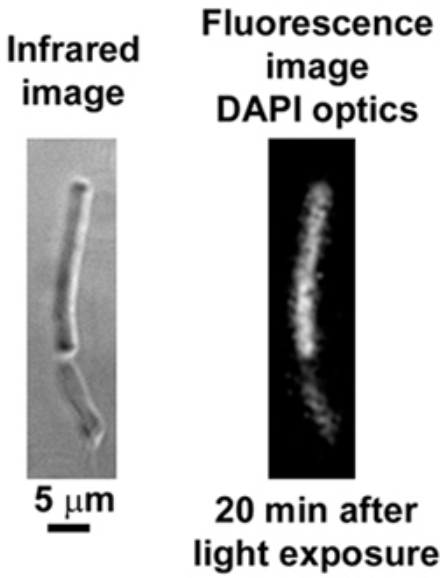

Figure 5. Mouse rod with retinol. This is a healthy mouse rod photoreceptor showing significant outer segment fluorescence after light exposure due to the formation of vitamin A. The ellipsoid regions of mouse rod photoreceptors do not show a strong fluorescence signal.

Discussion

If healthy isolated cells are not obtained, the problem lies either with the isolation or health of the retina or with its chopping. Typically, after removing the front of the eye and the vitreous, the retina readily lifts off the pigment epithelium. If it does not, try to peel it off starting from the periphery of the eyecup. If it is still difficult to separate, a likely possibility is that the animal has not been dark-adapted for an adequate period of time, or the red light is too bright. Ensure proper dark-adaptation for the animal, and dim the red light. The presence of rod photoreceptors in the retina can be easily ascertained by checking the color of the isolated retina: cut a small piece of retina and transfer it to a separate Petri dish, which can then be viewed under room lights. A piece of retina containing rod photoreceptors has a bright red color (due to rhodopsin) that fades rapidly. A colorless piece would indicate the absence of rhodopsin, and hence of rod photoreceptors. This might be due either to improper separation of the retina from the pigment epithelium or to an unhealthy retina. In such case, you should ensure the health of the animals and their proper dark adaptation. If a healthy retina is obtained but not healthy isolated cells, then the problem is most likely with the chopping. A fine chopping is critical: if the chopping is too coarse, or the retina becomes unstuck, it results mostly in pieces of retina instead of isolated cells. Good chopping typically results in a “cloud” of cells appearing in the solution.

Fluorescence imaging of single photoreceptors cells can use endogenous cell fluorophores such as NADH, FAD or vitamin A, as well as fluorescent dyes sensitive to different factors, to probe a wide range of physiological processes in real time. The method can be applied to many different species, including amphibians such as salamander (*Ambystoma tigrinum*)[17,18] and frog (*Rana pipiens*)[20], lizards (*Gecko gecko*)[21],

fish (zebrafish, *Danio rerio*)[11], and mouse (*Mus musculus*)[22]. The extension of the method to mouse cells allows the study of different types of genetically modified animals.

Disclosures

No conflicts of interest declared.

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