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Microfluorometric measurement of the formation of all-*trans* retinol in the outer segments of single isolated vertebrate photoreceptors

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Abstract

The first step in the detection of light by vertebrate photoreceptors is the photoisomerization of the retinyl chromophore of their visual pigment from 11-*cis* to the all-*trans* configuration. This initial reaction leads not only to an activated form of the visual pigment, Meta II, that initiates reactions of the visual transduction cascade, but also to the photochemical destruction of the visual pigment. By a series of reactions termed the visual cycle, native visual pigment is regenerated. These coordinated reactions take place in the photoreceptors themselves as well as the adjacent pigment epithelium and Müller cells. The critical initial steps in the visual cycle are the release of all-*trans* retinal from the photoactivated pigment and its reduction to all-*trans* retinol. The goal of this monograph is to describe methods of fluorescence imaging that allow the measurement of changes in the concentration of all-*trans* retinol as it is reduced from all-*trans* retinal in isolated intact salamander and mouse photoreceptors. The kinetics of all-*trans* retinol formation depend on cellular factors that include the visual pigment and photoreceptor cell type, as well as the cytoarchitecture of outer segments. In general, all-*trans* retinol forms much faster in cone cells than in rods.

Keywords

retina; rod; cone; visual pigment; rhodopsin; visual cycle

1. Introduction

Absorption of incoming light by the visual pigment of vertebrate photoreceptors isomerizes its retinyl chromophore from 11-*cis* to all-*trans*. This photoisomerization results in the formation of Meta II, an enzymatically active visual pigment conformation, and begins the transduction of light to an electrical signal that can be transmitted to the brain (see (1) for review). The all-*trans* chromophore is then removed and recycled to reform 11-*cis* retinal that can be used to regenerate the pigment (see (2-5) for reviews). The first steps of this process take place in the photoreceptor outer segment and culminate in the generation of all-*trans* retinol, which is formed through the reduction of the all-*trans* retinal released from the photoactivated visual pigment. The conversion of all-*trans* retinal to all-*trans* retinol is

catalyzed by the enzyme retinol dehydrogenase (RDH) and requires metabolic input in the form of NADPH. In the case of rods, all-*trans* retinol is transferred to neighboring cells of the retinal pigment epithelium (RPE) where it is esterified by lecithin retinyl acyltransferase. The resultant ester is the substrate for the Rpe65 isomerohydrolase that generates 11-*cis* retinol, which is then oxidized to 11-*cis* retinal and translocated to the photoreceptor outer segments; there, it condenses with opsin left behind following the release of all-*trans* retinal to regenerate the visual pigment. In the case of cones, an additional pathway involving the Müller cells can generate 11-*cis* retinal and regenerate the visual pigment independently of the RPE (6,7). The transfer of all-*trans* retinol from the outer segments appears to occur through mass action (8-11), and allows for the recycling of the chromophore to make fresh 11-*cis* retinal. In the rest of the chapter, where unspecified, retinal and retinol refer to their all-*trans* isomers.

Several steps comprise the pathway that forms all-*trans* retinol in the outer segment after the photoisomerization of the visual pigment's 11-*cis* retinyl chromophore. The initial step is the hydrolysis of the Schiff base bond between the chromophore and the visual pigment protein and the release of all-*trans* retinal. The released all-*trans* retinal may be sequestered inside the disks in the form of a Schiff base with phosphatidylethanolamine; it can then become available for reduction after it has been transferred to the cytosol by the ABCA4 transporter (12). The final step is the reduction of all-*trans* retinal by RDH, a reaction that uses NADPH as a co-factor. Because of the substantial amount of chromophore present in photoreceptor outer segments (3-4 mmol/L (13)), the kinetics of RDH and the availability of NADPH may have a significant impact on the overall kinetics of retinol formation. In summary, the kinetics of retinol formation depend on the rate of retinal release, local NADPH availability, retinal access to RDH, RDH kinetics, and retinol elimination rate (that depends on outer segment morphology).

The kinetics of the formation of retinol after light excitation can be monitored in the outer segments of single photoreceptors from its intrinsic fluorescence, which has been shown to provide a good measure of its concentration (9,14). Such single cell fluorescence measurements with their high sensitivity and time resolution provide a unique window for examining the different steps in the formation of retinol. They have been used to study the formation of retinol in the rod and cone photoreceptors from several vertebrate species, including tiger salamander (*Ambystoma tigrinum*), grass frog (*Rana pipiens*), lizard (*Gecko gecko*), and mouse (*Mus musculus*) (8-10,14-16). From among these species, tiger salamanders and mice offer several distinct advantages as model systems for the study of retinol formation. From the salamander retina, one can obtain different types of rods and cones that include two types of rods, green- and blue-sensitive, and three types of cones, red-, blue- and UV-sensitive (10,17). These salamander cells are large, robust, and can survive for several hours after isolation from the retina, facilitating the experimental manipulations for single cell imaging. Their large size also allows measurement of the time course and kinetics of retinol formation in local regions of the outer segments. Salamander photoreceptors utilize different pigment types as well as the same pigment in different cell types (10,18). Thus, they allow the comprehensive examination of the dependence of retinol formation on cell type, visual pigment type, and outer segment architecture. The mouse retina offers complementary advantages. It is dominated by a single cell type, the rods, allowing comparisons with biochemical measurements from isolated retinas (14) and the eyes of whole animals. Furthermore, the availability of genetically modified animals offers the opportunity to probe specific enzyme involvement and disease relevance.

Figure 1 shows a diagram of the setup that is used for such measurements, and includes an inverted microscope, fitted with a near UV fluorescence excitation light source and a high sensitivity camera. The orientation and function of these separate components is described

below (Methods) and in the figure legend. An experiment begins with placement of dark-adapted photoreceptor cells in the experimental chamber. After an initial fluorescence measurement in their dark-adapted condition, the cells are illuminated to ensure that virtually all of the visual pigment chromophore has been isomerized to the all-*trans* conformation. The reason behind this is that the UV light used to excite the fluorescence of retinol is also absorbed by the visual pigment and thus isomerizes the 11-*cis* chromophore. So, to avoid having each measurement of fluorescence initiating the reactions that lead to additional retinol formation, each experiment begins with photoactivation (bleaching) of virtually all of the visual pigment. Figure 2 shows an experiment performed on an isolated salamander green-sensitive rod photoreceptor in this way. Here it can be seen that, following quantitative bleaching of the visual pigment, retinol fluorescence increases within the outer segment, assuming a maximal value at about 30 min, and declining slowly thereafter. The data presented in Figure 3 illustrate a similar experiment performed on an isolated salamander red-sensitive cone cell. In this case, retinol fluorescence increases rapidly after bleaching, reaching a maximal value after ca. 1 min. Subsequently, it declines at a much faster rate than in the rod. Finally, Figure 4 presents an experiment with an isolated mouse rod, performed at 37 °C.

2. Materials

A dark room is necessary for the fluorescence imaging setup. The same room can be used for dark-adapting animals and for dissection. An area of ~100-150 sq.ft. is sufficient. A revolving door for entering is convenient, but a thick black curtain is also adequate.

2.1 Photoreceptor cell preparation

1. Red lights for the dark room. These are obtained from photographic equipment stores. A good choice is adjustable Kodak safelights fitted with Kodak Wratten #2 filters. If individual red bulbs are used, an appropriate choice is the Delta 1 Jr. Safelight. It is best to keep the red lights as dim as possible.
2. Larval tiger salamanders (*Ambystoma tigrinum*) are obtained from approved vendors (The Sullivan Company, Nashville, TN; Kons Scientific, Germantown, WI). Salamanders are usually available throughout the year; however, check with the supplier well in advance for availability.
3. Wild type mice (*Mus musculus*) are obtained from approved vendors (The Jackson Laboratory, Bar Harbor, ME; Harlan Laboratories, Indianapolis, IN). Genetically modified animals can be obtained from appropriate sources.
4. Salamander Ringer's with composition: 110 mM NaCl, 2.5 mM KCl, 1.6 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, pH = 7.55. The pH should be adjusted to the final value with NaOH [see Note 1]. The Ringer's solution can be kept well-sealed at room temperature for months. At the time of the experiment, glucose (5 mM) [see Note 2] and delipidated bovine serum albumin (concentration 0.01%) are added [see Note 3].

¹It is critical that the buffer composition is accurate within a few percent. The osmolarity of the solution affects the function and viability of the cells, especially the murine ones.

²We have obtained the same results using a Ringer's solution with a slightly different composition: 110 mM NaCl, 2.5 mM KCl, 1.6 mM MgCl₂, 1.0 mM CaCl₂, 10 mM glucose, 10 mM HEPES, pH 7.8.

³The addition of bovine serum albumin is not strictly necessary, but we have found that it appears to improve the viability of the cells. At this concentration, albumin does not affect the removal of retinol from the outer segment.

5. Mammalian Ringer's with composition: 130 mM NaCl, 5 mM KCl, 0.5 mM MgCl₂, 2 mM CaCl₂, 25 mM hemisodium-HEPES, pH = 7.40. At the time of the experiment, glucose (5 mM) is added.
6. Stock glucose solution, 1 M. This solution has to be kept at -20 °C to avoid bacterial growth.
7. Dissecting microscope.
8. Infrared (IR) light source. This can be a home-made infrared-LED-based system. Alternatively, an infrared safelight (FJW Optical Systems, Inc.) can be used.
9. Two infrared image viewers are attached to the dissecting microscope eyepieces (FJW Optical Systems, Inc.). The same company provides the components necessary for attaching the viewers to the dissecting microscope eyepieces.
10. Infrared viewer with illuminator (FIND-R-SCOPE Infrared Viewer with Illuminator Model 85100A; FJW Optical Systems, Inc.).
11. Petri dishes. 35 mm plastic (Falcon) dishes (Fisher Scientific).
12. Plastic transfer pipettes, 5 mL (Fisher Scientific).
13. Filter paper (Wratten; Fisher Scientific).
14. Dissecting tools (Fine Science Tools or Roboz Surgical Instruments). One pair of delicate iris scissors, straight, 11.5 cm long. One pair of extra fine Bonn scissors, curved, 8.5 cm long. At least two pairs of fine Dumont forceps, numbers 5 or 7. A couple of pairs of inexpensive student Dumont forceps (number 5) are also useful during the dissection. One razor blade holder/breaker. Pithing needles.
15. Sylgard 184 elastomer kit (Essex, Charlotte, NC).
16. Metal cutter (local hardware store or Fisher Scientific).
17. Double-edge razor blades, "Personna Double Edge Platinum Chrome" (local drug stores or pharmacies).
18. Experimental chambers. These can be 35 mm culture dishes with a 12 mm chamber (Warner Instruments, Hamden, CT).
19. Coating solution for chambers: 0.01% Poly-L-Ornithine solution or 0.1% Poly-L-Lysine solution (Sigma-Aldrich Chemical Company, St. Louis, MO). The Poly-L-Lysine solution is diluted with distilled water to a final 0.01% concentration.
20. Three light-tight boxes that can accommodate 2-3 of 35 mm Petri dishes each.
21. Fiber optic illuminator and longpass (>530 nm) filter for bleaching the cells (Edmund Optics, Barrington, NJ) [see Note 4].

2.2 Fluorescence Imaging

1. An inverted microscope with a light train that can be used for epifluorescence measurements. An inverted microscope is best, as it allows the use of high numerical aperture oil-immersion objectives that are critical for fluorescence measurements from single cells. The microscope should have a port for the CCD

⁴This long pass filter can be used for bleaching the visual pigments of the salamander green-sensitive rods and red-sensitive cones, and mouse rods. These three cell types comprise the overwhelming majority of the cells isolated from the retinas of these two species. For experiments with the salamander blue-sensitive rods and cones and UV-sensitive cones, a much more demanding technical approach is required, including different filters for bleaching the visual pigments of these cells (see section 3.2 paragraph 5).

camera, and the microscope optics should allow a setting for 100% of the fluorescence signal to be directed to the camera port.

2. Table for the microscope: a 3 ft × 3 ft vibration isolation table (Newport, Irvine, CA, or TMC, Peabody, MA), but a solid table of similar size is sufficient. The height of the table should allow the legs of an experimenter sitting in a chair to go under it.
3. The microscope should be placed in a light-tight enclosure (a “cage”), that allows access to microscope controls for the experimenter. A solid, sturdy frame that can support the weight of the cage is essential and can be constructed from wood or metal. The frame should rest on the floor and reach a height a few inches above the top of the microscope; it should have a couple of inches clearance from the sides of the table to allow bringing in optical and electronic cables. The left and right sides, top, and back of the cage can be constructed from aluminum plates screwed on or nailed to the frame and should begin 1-2 inches below the surface of the microscope table [see Note 5]; the inside surface of these plates should be painted black [see Note 6]. The front of the cage should be left open and covered with a double curtain made from black cloth. The inside curtain can be thin and should have two slits to allow the hands of an experimenter to control the microscope. A wooden horizontal rod attached at the bottom helps to roll the curtain up or down and keep it in place during experiments [see Note 7]. The outside curtain should be thick and drop to 1-2 inches below the surface of the microscope table [see Note 8]. If a vibration isolation table is used, a hand rest attached to the frame and placed about 1 inch above the height of the microscope table is very helpful [see Note 9].
4. Microscope stage: it should include an adaptor in which the experimental chambers readily fit. Such an adaptor is usually available from the microscope manufacturer, as the size of the experimental chambers is fairly standard.
5. Perfusion components. Slow perfusion of the cells during the course of the experiment improves their viability. A gravity-fed flow rate of 0.1-0.5 mL/min is appropriate; higher rates might disturb or even dislodge the cells [see Note 10]. The solution is continuously removed from the chamber and into a beaker below the microscope stage through a passive, wick-facilitated system. Alternatively, the solution can be removed through suction. In this case, use a 14 gauge needle connected to the vacuum line through a 3-mL syringe and plastic tubing to remove the solution from the chamber [see Note 11].
6. Infrared light source for microscope: an infrared filter (>850 nm) in front of the microscope’s transmitted light source can be used to provide the infrared light for

⁵You can place solutions for perfusion on the top of the cage (instead of inside). In that case, the top plate should have a 2-3 inch diameter hole to allow the tubes carrying the solutions to enter the cage.

⁶You can improve the light-tightness of the enclosure by attaching a 5-inch skirt made of black cloth around the bottom edges of the plates.

⁷The width of the inside curtain should be the same as the width of the frame; its top side should be permanently attached to the frame. The horizontal rod at the bottom is used to roll the curtain up or down as needed.

⁸The outside curtain must fully cover the inside one, so its width is longer than the frame. It is not permanently attached to the frame, and is kept in place during experiments with Velcro strips at the top, and the right and left sides. Place the Velcro on the frame and the curtain, so that the curtain is securely held but is also loose at the bottom. This is necessary to allow the hands of the experimenter to go under the outside curtain and through the slits of the inside one to reach the microscope.

⁹A ½-inch thick piece of wood that is about 5 inches deep and runs the length of the front side of the cage is adequate.

¹⁰The flow rate can be controlled by adjusting the height of the reservoir containing the solution or by compressing the plastic tubing that brings the solution from the reservoir to the chamber.

¹¹Placement of the needle is critical for avoiding complete removal of the solution in the chamber and drying the cells. A disadvantage of the suction system is that it is difficult to avoid pulsation, because the height of the solution in the chamber tends to fluctuate. Because the suction generates a sound however, it allows monitoring of perfusion from the outside of the light-tight cage. Cessation of the sound is an important warning sign, indicating potential flooding on the microscope stage.

viewing the dark-adapted cells on the microscope stage. The dark-adapted cells on the microscope stage have to be protected from any visible light leak from this source. One possible arrangement is for the light source to be placed outside the cage and the light be brought in via a light guide. Another option is to use an infrared LED (wavelength >850 nm) placed behind the condenser. In either case, it is necessary to be able to easily switch the infrared light on and off.

7. Objective lens: a high magnification (at least 40×) and numerical aperture (at least 1.3) oil-immersion lens is necessary. Use a lens appropriate for epifluorescence measurements [see Note 12].
8. Lens immersion oil: this should be of high quality and have low intrinsic fluorescence. A suitable one is Cargille Type FF Nonfluorescing immersion oil (Fisher Scientific).
9. Lens cleaning paper and solution (Fisher Scientific).
10. Stage, solution and objective lens heating. Experiments with mouse photoreceptors have to be carried out at 37 °C, necessitating heating the experimental chamber, the incoming solution, and the objective lens. A variety of heated stages compatible with the experimental chamber are available (Warner Instruments), along with a separate assembly for heating the incoming solution, heated jacket for the objective lens [see Note 13], and temperature controllers for each component. The temperature of the solution in the experimental chamber is measured independently and is maintained at 37 °C by adjusting the temperatures of the stage, the incoming solution, and the lens.
11. Excitation light source. A Xenon continuous arc light source (Sutter Instrument Company, Novato, CA; or Cairn Research Ltd., Faversham, UK) is used to provide the light to excite the fluorescence of retinol. An electronic shutter (Uniblitz; Vincent Associates, Rochester, NY) is placed in the light path to control the exposure of the cells to the excitation light [see Note 14]. The Xenon light source is placed on the floor under or next to the microscope table [see Note 15] and the excitation light is brought to the microscope port with a light guide. Adaptors for connecting the light guide to the light source and the microscope port are available (Sutter Instrument Company). The casing for the light source should have slots for neutral density filters to attenuate the excitation light intensity as necessary.
12. Neutral density filters and holders. The filter holders should fit into the slots in front of the excitation light source. Neutral density filters of 1-log unit (attenuation to 10%) and 2-log unit (attenuation to 1%) are necessary (Chroma Technology, Rockingham, VT).
13. Filters for retinol fluorescence measurement: filter set 11000v3 (Chroma Technology) includes parts D350/50× for excitation (bandpass filter centered at 350 nm with bandwidth of 50 nm), 400DCLP for dichroic mirror (reflects light <400 nm), and E420LPv2 for emission (longpass filter >420 nm) [see Note 16].

¹²The high numerical aperture is necessary to collect as much fluorescence signal as possible. A 100× lens might be somewhat more helpful especially for visualizing the small mouse photoreceptor cells, but because of the smaller field of view it makes it more difficult to find a cell. In our experience, an oil-immersion 40× lens with 1.3 numerical aperture (for example the Zeiss Plan Neofluar or even the Fluor) is perfectly adequate.

¹³The objective lens is a major heat sink and heating it helps to stabilize the temperature in the experimental chamber. Temperature fluctuations in the lens change the temperature of the immersion oil, resulting in changes in its refractive index and focus drift.

¹⁴The shutter is essential to avoid photobleaching of retinol. The cells should be exposed to the excitation light only for image capture.

¹⁵Do not crowd or try to cover the Xenon light source to reduce light leak. The lamp generates a lot of heat and good ventilation is necessary to avoid overheating.

14. High sensitivity CCD camera. Adequate sensitivity of the CCD camera is critical for successful imaging of retinol fluorescence in living cells. Suitable cameras include CoolSNAP HQ2 Monochrome (Photometrics, Surrey, BC), Sencam QE (Cooke Corporation, Auburn Hills, MI), or Orca-285 (Hamamatsu Photonics, Hamamatsu-City, Japan). An image intensifier (VS4-1845; OPELCO Inc., Dulles, VA) coupled to the camera can further improve detection but is expensive. The same camera is used in live mode with infrared illumination to find a dark-adapted cell to begin an experiment.
15. Image acquisition and analysis software. The software coordinates the function of the different hardware components to acquire images with user-specified parameters (for example, exposure time). Suitable packages include Slidebook (Intelligent Imaging Innovations, Denver, CO) and Openlab (Improvision Inc., Waltham, MA). The software package includes routines for image analysis.
16. Computer. The image acquisition and analysis software is installed in a computer that controls all hardware components. Consult with the software provider for appropriate computer specifications.
17. Computer monitor. Larger monitors are easier for the user, so the size of the monitor ultimately depends on the available space next to the microscope table. During an experiment, the screen should be covered with transparent red plastic (gel sheet Roscolux #27, Medium Red; theater lighting companies) to minimize light in the room.
18. Surge protectors for all electrical and electronic components. It is essential that the Xenon arc light source have its own separate surge protector. Other components can share a surge protector.

3. Methods

3.1 Photoreceptor cell preparation

3.1.1 Dishes, chambers, and razor blades

1. Coat the bottoms of 35 mm Falcon Petri dishes with Sylgard elastomer. Prepare the elastomer according to the instructions on the box and pour a small amount in each dish to cover its bottom with a thick layer. Replace the covers on the dishes and store them. The elastomer will harden over a period of a few days and the dishes will be ready.
2. Coat the bottoms of the experimental chambers with 0.01% poly-L-lysine or poly-L-ornithine; 200 μ L of solution per chamber is enough. Cover the chambers with a paper towel to protect them from dust and let them sit until dry. Wash them with distilled water and keep them upside-down to dry. Store in a closed box and use within 2 weeks.
3. The chambers can be re-used. After an experiment, wash the chamber with 100% ethanol to remove the oil (from the oil-immersion lens) on the outside and the cell debris on the inside of the chamber. Use cotton-tipped applicators to gently scrub the bottom of the chamber to remove the debris. Wash with distilled water and let dry.

¹⁶The absorption maximum of retinol is 325 nm, and its fluorescence emission maximum is ca. 480 nm (15). The glass optics used (lenses and light guide) transmit poorly <350 nm, hence the use of the particular excitation filter. Use of very expensive quartz optics should allow efficient excitation with 325 nm light, but we have no direct experience with such a system. The dichroic mirror and the longpass emission filter are selected to collect most of the fluorescence emission.

4. Prepare several small razor blades by cutting each double-edged blade into 8 pieces with the metal cutter.

3.1.2 Isolated retinas

1. Keep the animals healthy and clean, feed them and provide veterinary care [see Note 17].
2. Dark-adapt an animal in a ventilated container (for example, for salamanders, a suitably modified bucket) in the dark-room for at least 2-3 hours before beginning experiments.
3. Immediately before the experiment, add the appropriate glucose and/or bovine serum albumin concentrations. Use this Ringer's for experiments. Discard any leftover solution at the end of the day, as it might grow bacteria.
4. Pour some of the Ringer's solution to two 35 mm Petri dishes and keep them close to the dissecting microscope.
5. Sacrifice the animal under dim red light.
6. Enucleate the eyes using the long scissors and the student Dumont forceps.
7. The rest of the procedures are carried out under the dissecting microscope using infrared light. Use the infrared viewer with illuminator, if you need to find something outside the field of view of the microscope.
8. Remove any leftover muscle and skin tissue from the eye using the long scissors and the student Dumont forceps.
9. Tape a small piece of filter paper on the dissecting microscope stage and place the eye on it [see Note 18]. Remove the anterior part of the eye, leaving the vitreous in the eye cup. Use the short scissors to make an incision and cut around just behind the ora serrata.
10. Transfer the eyecup into one of the Petri dishes filled with Ringer's. Carefully remove the vitreous using the fine forceps.
11. Under the infrared light, the retina is now visible against the dark background of the retinal pigment epithelium. Gently separate the retina from the epithelium; it will remain attached to the eyecup at the optic nerve. With the fine forceps reach underneath the retina and pinch it off at the point of attachment. Separate the retina fully from the eyecup [see Note 19].
12. Using a plastic pipette, draw some solution containing the retina and transfer it to the other, clean Petri dish. It can be kept there in a light-tight box for a few hours [see Note 20].

¹⁷Do your best to ensure the health of the animals, because the health of the cells depends on it.

¹⁸It is not necessary to use filter paper, but we have found that it helps for handling the small salamander and mouse eyes by keeping them in place.

¹⁹Sometimes it is difficult to separate the retina from the pigment epithelium. Slowly peel off starting from the periphery. If you still cannot separate the retina, a likely possibility is incomplete dark adaptation, which could be caused by too bright a red light. Ensure that the animal is dark-adapted properly, and dim the red light.

²⁰It is a good idea to cut a small piece of retina and transfer it to a separate Petri dish. Under room lights this piece of retina should be a bright red color (due to rhodopsin) that fades rapidly. The red color indicates the presence of rod outer segments and confirms that you have obtained a healthy retina. Lack of red color indicates either an unhealthy retina or a failure to separate the rod outer segments from the retinal pigment epithelium. In such case, you should ensure the health of the animals and proper dark adaptation.

3.1.3 Isolated living photoreceptor cells

1. Bring pipettes, coated chambers, Sylgard-covered dishes close to the dissecting microscope. Grab a piece of razor blade with the blade holder, with the edge of the blade at approximately 45° angle to the holder. All subsequent procedures are carried out under the dissecting microscope using infrared light.
2. Using the small scissors, cut the retina into 2-3 pieces. With a plastic pipette, draw some solution containing a piece and transfer it into a Sylgard-covered dish. The final volume of the solution in that dish should be about 250 µL.
3. With the fine forceps flatten the piece of retina on the Sylgard surface, keeping the photoreceptor side up. Using the razor blade, chop the piece in one direction; repeat 3-4 times, then rotate the dish 90° and chop again 3-4 times. Repeat the whole procedure until you see a “cloud” of dissociated cells. It is important to chop finely, while keeping the piece of retina stuck to the Sylgard. If the chopping is too coarse, or the retina becomes unstuck, one gets mostly pieces of retina instead of isolated cells.
4. After finishing the chopping, transfer 200 µL of the solution to an experimental chamber. Keep the chamber with the isolated cells in a light-tight box.
5. Wait for 10 min for the cells to settle, then add 2-3 mL of Ringer's. The cells can now be taken to the microscope stage for the experiment [see Note 21].

3.2 Fluorescence imaging measurements

1. Bring the fiber optic cable of the illuminator to be used for bleaching the cells inside the light-tight cage and above the microscope stage; secure it so that its end is at a distance of about 2 inches from the nose of the objective lens. The nose of the lens should be at the center of the illuminating beam to ensure bleaching of the cells.
2. Put immersion oil on the objective lens [see Note 22].
3. Bring a dish over to the stage with the IR viewer using infrared light from its illuminator [see Note 23]. Bring the perfusion components to their positions and begin perfusion.
4. Close the curtains to the light-tight cage surrounding the microscope. The preparation should now be in darkness.
5. Make sure that all electronic equipment is switched off, and only then turn on the Xenon lamp [see Note 24]. Next, turn on the rest of the equipment, including camera, computer, and, for mouse experiments, the heating components. Last, turn on the monitor covered with red plastic.

²¹Before you embark on actual experiments, you need to ensure that the chopping and the cell density have been optimized. A very high cell density will result in cells settling on top of each other, disallowing an experiment. A very low density might result in failure to find a cell for experiment. Check your isolated cell preparations under the infrared illumination of the microscope, and adjust chopping and density until you can regularly obtain isolated intact cells, that have settled without cells above or below them.

²²At the end of the day, remove any residual oil on the lens with lens paper. With use, oil seeps under the lens. Remove and clean the lens regularly with cleaning fluid and lens paper. You can use cotton-tipped applicators to clean the back surface of the lens.

²³After the first experiment of the day, the equipment has been switched on. If you need to switch off the Xenon lamp to place a second dish on the microscope stage, then you will need to switch off all pieces of equipment (see Note 24 below). However, with the Xenon lamp on the floor, the light leaking from it should not reach the microscope stage inside the cage. If this is indeed the case, switch off only the computer monitor, and open the light-tight box containing the chamber with the cells inside the cage. Continue as with the first dish of the day.

²⁴When the Xenon lamp is turned on, it could generate a voltage spike that might damage other electronic equipment.

6. Turn on the microscope IR illumination and, with the camera in live mode, move the stage and find a cell. When working with isolated salamander cells, the overwhelming majority of the cells are green-sensitive rods and red-sensitive cones [see Note 25].
7. Only outer segments with attached ellipsoids can generate retinol. Among those, it is best to use whole intact cells (with outer segment, ellipsoid, and nucleus).
8. Carry out preliminary measurements to determine proper focus for fluorescence. Determine what the cell should look like under IR illumination to be in focus for retinol fluorescence. Make sure that the retinol signal is well above background and does not saturate the camera. Perform initial measurements with rod cells to measure the time-course of fluorescence production. Carry out tests after a post-bleach period at which time maximum retinol fluorescence is observed. Ensure that the measuring light does not photobleach retinol: make repeated measurements (about 10) of fluorescence in rapid succession. Any significant diminution (more than 0.5% per individual measurement) of the fluorescence signal can be attributed to retinol photobleaching. If significant photobleaching is observed, use the neutral density filters to attenuate the excitation light intensity and reduce the exposure time. With a CCD camera that has adequate sensitivity, using 1-10% of the Xenon lamp intensity and 100-500 ms exposure times should provide a clear retinol fluorescence signal without significant photobleaching [see Note 26].
9. For an experiment, find a cell under IR, center it in the field, and adjust the focus for fluorescence measurement. Switch off the IR and capture a fluorescence image. Turn on the fiber optic illuminator and bleach the cells on the microscope stage. Use 1 min illumination for rods and 10 sec for cones. Switch off the illuminator and capture another fluorescence image (for rods) or capture a series of images with a time delay acquisition routine (for cones) [see Note 27]. Switch on the IR to check the focus and continue, capturing images at specified times after bleaching. Remember to switch off the IR before capturing a fluorescence image.
10. Keep in mind that the focus drifts and that the cell might move slightly. Refocus for each measurement (except for the one measurement immediately after bleaching, when speed is of the essence).

3.3 Analysis of fluorescence imaging data

1. Use the software to define regions of interest (ROI) in the outer segment and in the background.
2. Use the ROIs to measure average fluorescence intensity for outer segment and background regions.

²⁵Blue-sensitive salamander rods and blue- and UV-sensitive cones are each morphologically distinct and can be distinguished from the green-sensitive rods and red-sensitive cones (17). For experiments with these types of cells however, it is best to independently corroborate their identity by measuring their spectral sensitivity. Such measurements require additional equipment (10).

²⁶An important additional parameter for image acquisition is image binning. Binning improves the signal-to-noise ratio at the expense of resolution by grouping the output of camera pixels. With the specified hardware components, and exposure times 100-1000 ms, we find that 2×2 and 4×4 binning provide images with adequate resolution and good signal-to-noise ratio.

²⁷In cone outer segments, retinol fluorescence increases rapidly after bleaching, so it is necessary to employ a time delay image acquisition routine to capture a series of images in rapid succession. This image acquisition routine is an option provided by the software and is necessary for capturing images with time delays less than 1 min. For longer time delays between images, single image capture is generally better. Using a time delay routine for an experiment lasting for more than 5 min, although not impossible, is usually unsuccessful, because the focus drifts and the cell might move. Refocusing with the IR before each image capture is necessary for these longer experiments.

3. Subtract background fluorescence from that of the outer segment ROI to obtain the outer segment intensity due to the outer segment fluorophore. Obtain outer segment fluorescence intensity for each time point.
4. Continue analysis for your purposes. Example, subtract initial control value to obtain the outer segment fluorescence due to retinol. Alternatively, normalize over the initial control value, or, analyze kinetics according to different models, etc.

References

1. Fain GL, Matthews HR, Cornwall MC, Koutalos Y. Adaptation in vertebrate photoreceptors. *Physiol Rev* 2001;81:117–151. [PubMed: 11152756]
2. Imanishi Y, Lodowski KH, Koutalos Y. Two-photon microscopy: shedding light on the chemistry of vision. *Biochemistry* 2007;46:9674–9684. [PubMed: 17676772]
3. Lamb TD, Pugh EN Jr. Dark adaptation and the retinoid cycle of vision. *Prog Retin Eye Res* 2004;23:307–380. [PubMed: 15177205]
4. McBee JK, Palczewski K, Baehr W, Pepperberg DR. Confronting complexity: the interlink of phototransduction and retinoid metabolism in the vertebrate retina. *Prog Retin Eye Res* 2001;20:469–529. [PubMed: 11390257]
5. Saari JC. Biochemistry of visual pigment regeneration: the Friedenwald lecture. *Invest Ophthalmol Vis Sci* 2000;41:337–348. [PubMed: 10670460]
6. Mata NL, Radu RA, Clemmons RC, Travis GH. Isomerization and oxidation of vitamin a in cone-dominant retinas: a novel pathway for visual-pigment regeneration in daylight. *Neuron* 2002;36:69–80. [PubMed: 12367507]
7. Wang JS, Estevez ME, Cornwall MC, Kefalov VJ. Intra-retinal visual cycle required for rapid and complete cone dark adaptation. *Nat Neurosci* 2009;12:295–302. [PubMed: 19182795]
8. Tsina E, Chen C, Koutalos Y, Ala-Laurila P, Tsacopoulos M, Wiggert B, Crouch RK, Cornwall MC. Physiological and microfluorometric studies of reduction and clearance of retinal in bleached rod photoreceptors. *J Gen Physiol* 2004;124:429–443. [PubMed: 15452202]
9. Wu Q, Blakeley LR, Cornwall MC, Crouch RK, Wiggert BN, Koutalos Y. Interphotoreceptor retinoid-binding protein is the physiologically relevant carrier that removes retinol from rod photoreceptor outer segments. *Biochemistry* 2007;46:8669–8679. [PubMed: 17602665]
10. Ala-Laurila P, Kolesnikov AV, Crouch RK, Tsina E, Shukolyukov SA, Govardovskii VI, Koutalos Y, Wiggert B, Estevez ME, Cornwall MC. Visual cycle: Dependence of retinol production and removal on photoproduct decay and cell morphology. *J Gen Physiol* 2006;128:153–169. [PubMed: 16847097]
11. Wu Q, Chen C, Koutalos Y. All-trans retinol in rod photoreceptor outer segments moves unrestrictedly by passive diffusion. *Biophys J* 2006;91:4678–4689. [PubMed: 17012326]
12. Weng J, Mata NL, Azarian SM, Tzekov RT, Birch DG, Travis GH. Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice. *Cell* 1999;98:13–23. [PubMed: 10412977]
13. Harosi FI. Absorption spectra and linear dichroism of some amphibian photoreceptors. *J Gen Physiol* 1975;66:357–382. [PubMed: 808586]
14. Chen C, Blakeley LR, Koutalos Y. Formation of all-trans retinol after visual pigment bleaching in mouse photoreceptors. *Invest Ophthalmol Vis Sci*. 2009 in press.
15. Chen C, Tsina E, Cornwall MC, Crouch RK, Vijayaraghavan S, Koutalos Y. Reduction of All-trans Retinal to All-trans Retinol in the Outer Segments of Frog and Mouse Rod Photoreceptors. *Biophys J* 2005;88:2278–2287. [PubMed: 15626704]
16. Kolesnikov AV, Ala-Laurila P, Shukolyukov SA, Crouch RK, Wiggert B, Estevez ME, Govardovskii VI, Cornwall MC. Visual cycle and its metabolic support in gecko photoreceptors. *Vision Res* 2007;47:363–374. [PubMed: 17049961]
17. Perry RJ, McNaughton PA. Response properties of cones from the retina of the tiger salamander. *J Physiol* 1991;433:561–587. [PubMed: 1841958]

18. Ma J, Znoiko S, Othersen KL, Ryan JC, Das J, Isayama T, Kono M, Oprian DD, Corson DW, Cornwall MC, Cameron DA, Harosi FI, Makino CL, Crouch RK. A visual pigment expressed in both rod and cone photoreceptors. *Neuron* 2001;32:451–461. [PubMed: 11709156]

Figure 1.

Diagram of the experimental apparatus. Bright-field infrared image of a rod and a cone photoreceptor is shown at the top and a fluorescent image of these cells is shown at the bottom. The fluorescent image was acquired before visual pigment bleaching.

Figure 2.

Formation of all-*trans* retinol in an isolated rod photoreceptor from a larval tiger salamander retina. (A) Retinol fluorescence increases in the rod outer segment after rhodopsin bleaching. a, infrared image of an isolated rod photoreceptor cell, b-g, fluorescence (excitation, 360 nm; emission, >420 nm) images of the cell; b, before bleaching; c, immediately after; d, 10 min; e, 30 min; f, 50 min; g, 90 min after bleaching. All fluorescence images are shown at the same intensity scaling. Bar is 10 μ m. (B) Rod outer segment fluorescence intensity values before ($t = -1$ min) and at different times after rhodopsin bleaching for the cell in (A). Bleaching was carried out from $t = -1$ to 0 min.

Figure 3.

Formation of all-*trans* retinol in an isolated cone photoreceptor from a larval tiger salamander retina. (A) Retinol fluorescence increases in the cone outer segment after visual pigment bleaching. a, infrared image of the isolated cone photoreceptor cell, b-g, fluorescence (excitation, 360 nm; emission, >420 nm) images of the cell; b, before bleaching; c, immediately after; d, 0.5 min; e, 1 min; f, 5 min; g, 30 min after bleaching. All fluorescence images are shown at the same intensity scaling. Bar is 10 μ m. (B) Cone outer segment fluorescence intensity values before ($t = -10$ sec) and at different times after visual pigment bleaching for the cell in (A). Bleaching was carried out from $t = -10$ sec to 0 min. The inset shows the same data as the main panel in an expanded time scale.

Figure 4.

Formation of all-*trans* retinol in an isolated rod photoreceptor from a c57bl/6 mouse. (A) Retinol fluorescence increases in the rod outer segment after rhodopsin bleaching. a, infrared image of an isolated rod photoreceptor with intact ellipsoid b-g, fluorescence (excitation, 360 nm; emission, >420 nm) images of the cell; b, before bleaching; c, immediately after; d, 10 min; e, 30 min; f, 50 min; g, 90 min after bleaching. All fluorescence images are shown at the same intensity scaling. Experiment carried out at 37 °C. Bar is 5 μ m. (B) Rod outer segment fluorescence intensity values before ($t = -1$ min) and at different times after rhodopsin bleaching for the cell in (A). Bleaching was carried out from $t = -1$ to 0 min.