Chapter 2

Rhodopsin Purification from Dark-Adapted Bovine Retina

Elise Blankenship and David T. Lodowski

Abstract

Structural and biophysical studies of rhodopsin have long depended upon the ready availability of bovine retina from the meat-packing industry and the relative ease of obtaining homogenous preparations of rhodopsin in the quantities and purities necessary for such study. Herein we present a modular purification methodology employing a combination of several strategies, beginning with sucrose gradient isolation of rod outer segments (ROS) from bovine retina, detergent solubilization of ROS, selective extraction of rhodopsin starting from this detergent-solubilized ROS, and further purification via size-exclusion chromatography, resulting in a preparation of high-purity rhodopsin at high concentration suitable for crystallization or other biophysical study.

Key words Rhodopsin, Purification, Zinc extraction, Size-exclusion chromatography

1 Introduction

With its initial characterization in the 1870s by Boll and Kühne [1], the retina (and extracts thereof) has formed the basis of much that is understood about G protein-coupled receptor structure and function, providing much of the raw material from which biochemical, biophysical, and structural work have initiated. Critical in these studies have been the high concentration of rhodopsin present in the rod outer segment (ROS) of retinal rod cells and the ability of mechanical disruption to shear these ROS from the residual retinal tissue. Isolation of these ROS can be achieved through the use of sucrose gradient centrifugation resulting in an ~80 % pure preparation of rhodopsin imbedded in ROS membranes [2]. Detergent extracts of these ROS membranes were later used to purify the rhodopsin utilizing concanavalin A (lectin) affinity, hydroxyapatite chromatography, size-exclusion chromatography, or ion exchange affinity chromatography [3–7]. However, it was not until the discovery that alkyl glucoside detergent extracts of these ROS preparations could be further purified by treatment with divalent cations [8], resulting in the precipitation of opsin

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(apoprotein rhodopsin) and other membrane proteins, while the majority of ground-state rhodopsin remained in solution, that diffracting crystals of rhodopsin in its ground-state were obtained [9]. This purification was critical in obtaining the first structures of ground-state rhodopsin and still is utilized as an initial step in the purification of rhodopsin. Additional ground-state structures have employed a combination of concanavalin A (lectin) affinity chromatography followed by a secondary purification utilizing anion exchange chromatography and utilized C_8E_4 detergent rather than the alkyl glucoside detergents employed in previous ground-state studies [10]. Recognizing that these extractions still left a large amount of lipids, an undefined amount of detergent, and Zn^{2+} ions bound to the rhodopsin, additional purification with 1D4 antibody affinity chromatography was necessary to obtain diffracting crystals which were stable upon light exposure [11, 12].

Contemporary studies necessitating the isolation and reconstitution of rhodopsin complexes have routinely utilized combinations of the above methodologies [13]. Yields of 50–70 mg of >95–99 % pure rhodopsin are typical for preparations from 100 dark-adapted bovine retinas. It is unnecessary to analyze fractions via SDS-PAGE during the purification as the specific absorbance of rhodopsin at 500 nm is diagnostic of the presence of ground-state rhodopsin [14], and the ratio of this number to the total protein contained in the sample as measured by absorbance at 280 nm is indicative of purity [15, 16]. Furthermore, SDS-PAGE analysis yields little information on the fraction of active rhodopsin in the sample as it is insensitive to discerning between the apoprotein opsin (a, if not the, major contaminant in these preparations) and rhodopsin.

2 Materials

Prepare all solutions using ultrapure water (18.3 MΩ/cm at 25 °C) and analytical grade reagents. All buffers and stock solutions should be stored at 4 °C unless otherwise stated. We do not add sodium azide to the sucrose solutions, but it may be added at 0.05 % w/v if long-term storage of excess buffer is desired.

2.1 Bovine Eye Dissection

1. Scalpel and/or surgical scissors.
2. Blunt tip forceps.
3. Amber pill bottle(s) for retinal storage.
4. Aluminum foil.
5. Fresh bovine eyes: these can be procured by special request from a local slaughterhouse (*see* **Note 1**).

2.2 ROS Membrane Isolation

2.2.1 Required Supplies

1. Hydrometer(s) capable of measuring specific gravity spanning the range of 1.10–1.15 (Fisherbrand).
2. 50 ml polycarbonate capless tubes (Nalgene).
3. Tube rack for 50 ml tubes.
4. Cannula (14 G, 6 in. long, blunt point needle) (Cadence Science).
5. Disposable 10 and 30 ml syringes.
6. 250 ml glass graduated jar with Teflon seal in lid (Qorpak).
7. 250 ml graduated cylinder (three needed). We prefer polycarbonate graduated cylinders for use in the darkroom as they are less fragile than glass and easier to read than polypropylene cylinders.
8. 500 ml graduated cylinder.
9. Glass funnel large enough to sit on top of the 250 ml graduated cylinder.
10. Two $4 \times 4''$ gauze surgical sponges, unfolded to create an $8 \times 8''$ 4 ply liner for the funnel.
11. Beckman JA-17 or JA-20 rotor or equivalent rotor (*see* **Note 2**).
12. Beckman JS-13.1 rotor or equivalent rotor.
13. Beckman Avanti J26XP centrifuge or equivalent high-speed centrifuge located in a darkroom.
14. Quartz semimicro cuvette, 0.6 ml volume (Starna).
15. Electric pipette aid and disposable 10 ml pipettes for resuspension of pelleted crude ROS membranes (optional: 10 ml syringe and a cannula can be used for this task).

2.2.2 Required Buffers

The protocol presented here is suitable for large-scale purification, and buffer amounts are enough for 3–4 purifications of 100–150 retinas (*see* **Note 3**); smaller volumes of each buffer can be prepared for smaller preparations, although the time involved in making these solutions should discourage the preparation of just enough solution for a single preparation at a time.

1. Kühn's buffer: 67 mM $KH_{(x)}PO_4$, pH 7.0, 1 mM $Mg(C_2H_3O_2)_2$, 0.1 mM EDTA, and 1 mM DTT (optionally, sodium azide can be added at a concentration of 0.05 % to inhibit bacterial and fungal growth in the buffer during storage).
2. Kühn's buffer containing 45 % (w/v) sucrose.
3. Kühn's buffer containing sucrose to make the following specific densities: 1.10, 1.11, 1.13, and 1.15 (*see* **Note 4** and Table 3 for detailed instructions on how to make the sucrose gradient buffers required for this purification).

2.2.3 Determination of Rhodopsin Concentration

1. Rhodopsin determination buffer: 10 mM β-D-dodecyl maltoside (DDM) or 30 mM 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) detergent, 20 mM Bis tris propane pH 7.5, and 20 mM Hydroxylamine. This buffer can be prepared in advance and 5-10 ml aliquots can be stored at -20 °C until needed (*see* **Note 5**).

2.3 Rhodopsin Purification

2.3.1 Zinc Extraction

1. 500 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.3 stock solution.
2. 50 mM MES pH 6.3 (prepared from above stock).
3. $Zn(O_2CCH_3)_2 \cdot (H_2O)_2$ or $ZnCl_2$ (*see* **Note 6**).
4. N-β-D-nonyl-glucoside (NG) (Affymetrix) powder or 500 mg/ml stock (in 50 mM MES, pH 6.3) (*see* **Note 7**).

2.3.2 Dialysis for Zinc Removal and Detergent Exchange

Due to the fact that buffers containing high concentrations of zinc spontaneously, albeit slowly, form insoluble $Zn(OH)_2$, samples should be dialyzed soon after extraction to remove the majority of zinc; the use of mildly acidic buffers for the zinc extraction and dialysis minimizes the formation of $Zn(OH)_2$ (*see* **Note 8** for comments on detergent exchange during dialysis).

1. Dialysis buffer: 20 mM MES, pH 6.3, 6.5 mM NG, 200 mM NaCl, 10 mM EDTA, and 1 mM DTT.
2. Dialyzer membrane: Slide-A-Lyzer (Pierce) or Float-A-Lyzer (Spectrum) with at least a 20 kDa molecular weight cutoff to allow for ease of movement of detergent across the membrane (*see* **Note 9**). Dialyzer membrane should be wetted with water or dialysis buffer prior to loading.

2.3.3 Size-Exclusion Chromatography

Size-exclusion chromatography should be carried out at 4 °C using an HPLC or equivalent chromatography system which has been adapted to run under dark conditions. This is best accomplished with a chromatography refrigerator placed in a darkroom. In order to adapt the HPLC to operation in the dark, all non-red LED lights on the HPLC must be covered with foil tape (Nashua) or other lighttight coverings. It is best to further protect the sample from light by wrapping all tubing as well as the column in aluminum foil. If the HPLC cannot be controlled from outside of the darkroom, the monitor can be covered with a dark red filter (Roscolux Medium Red Filter) (*see* **Note 10** for details on filter material).

1. For dealing with the large volume of concentrated rhodopsin produced in these large-scale purifications, a large preparative gel filtration column such as a GE HiLoad 16/600 Superdex 200 pg with a column volume of 120 ml is suggested.

Table 1
Recommended detergents and concentrations for gel filtration and detergent exchange

Detergent	CMC (mM)	Suggested detergent concentration (mM)
n-Heptyl-β-D-glucoside (C_7G, HG)	70	70
n-Octyl-β-D-glucoside (C_8G, OG)	20	20–40
n-Nonyl-β-D-glucoside (C_9G, NG)	6.5	6.5–13
n-Decyl-β-D-glucoside (C_{10}G, DM)	2.2	4.4
n-Nonyl-β-D-maltoside (C_9M, NM)	6.0	12
n-Dodecyl-β-D-maltoside (C_{12}M, DDM)	0.17	1
Octyl glucose neopentyl glycol (OGNG)	1.02	2.0
Lauryl maltose neopentyl glycol (LMNG)	0.01	0.1–1.0

2. For smaller-scale purifications, we suggest either a GE 10/300 Superdex 200 or a Sepax SRT-C 10/300 column. Both of these columns have similar column volumes (~25 ml); however, the silica-based media in the Sepax columns has significantly better separation efficiency, resulting in tighter, more concentrated peaks, although this comes at the expense of reduced pH range over which the protein can be exchanged (pH 2.5–8.0).
3. Size-exclusion buffer containing detergent: 10 mM MES, pH 6.3, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, and 6.5 mM NG (or another suggested detergent in appropriate concentration; *see* Table 1). Filter through a 0.22 μm filter. If desired, buffer can also be degassed by sparging with argon or helium at this stage. Add dry detergent to at least the critical micelle concentration (CMC) of the detergent (*see* Table 1) and stir until fully dissolved. Ranges are presented only when multiple concentrations have been tested (*see* **Note 11**).

3 Methods

All procedures are carried out in a darkroom with only dim red light illumination. All buffers should be at 4 °C.

3.1 Procuring/ Dissecting Bovine Retina

Dissection of the retina should be performed as soon as possible after removal from the bovine carcass as the retina deteriorates over time and becomes less attached to the retinal pigmented

epithelium. Due to the time and monetary expense of bovine eye procurement and retinal dissection, when experimentally allowable, frozen dark-adapted bovine retinas are a time-saving and cost-effective source of rhodopsin (W.L. Lawson Co. or InVision BioResources). The purification of rhodopsin from these frozen tissues produces homogenously pure rhodopsin which is of sufficient quality for crystallization or other biophysical study. Gloves and adequate eye protection must be used for the retinal dissection. Ocular tissue must be discarded according to institutional rules for neurological material. A headlamp with a red filter or red LED light conveniently illuminates the workspace while keeping the undissected eyes and dissected retinas in the dark. *See* Fig. 1 for a schematic representation of the bovine eye and optimal cutting location on the eye to expose the retina (s*ee* **Note 12** for safety concerns when dealing with bovine eyes and other neurological material).

1. Arrange eyes "facing" upward in a glass or metal dish sitting in a container of ice; this ensures that the rear of the eye and retina is kept as cool as possible, slowing the detachment of the retina which complicates the dissection.
2. Before cutting into the eye, it may be necessary to pull back the fat and connective tissue which may slip forward over the eye after removal in order to reach the sclera and cornea of the eye for dissection.
3. Remove the cornea using a sharp scalpel or sharp surgical scissors (*see* **Note 13**) to puncture the sclera (white of the eye)

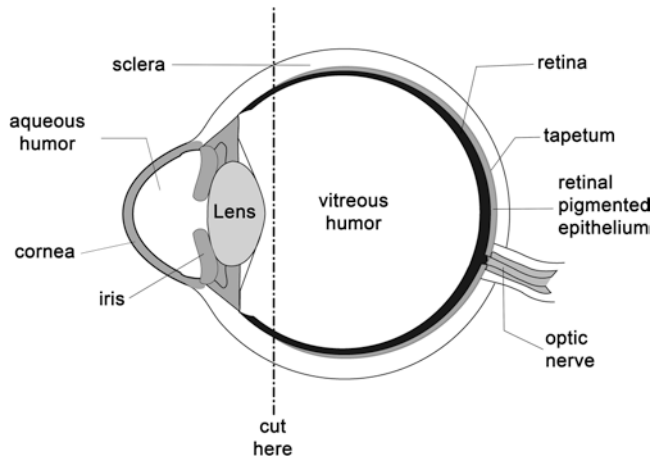

Fig. 1 Bovine eye anatomy and dissection guide. A bovine eye is shown in sagittal cross section with approximate location (*dotted line*) for the incision through the sclera to produce an eyecup for the retinal dissection. If the cut around the circumference of the eye is too close to the iris, it may be difficult to turn the eyecup inside out; conversely, simply cutting the eye in half equatorially will decrease the amount of retina harvested from each eye

close to the cornea (*see* Fig. 1), then slice around the circumference of the sclera forming an "eyecup." The eyecup should comprise the back 60–75 % of the eye.

4. Gently turn the eyecup inside out over your thumb and discard the lens and vitreous humor. If this is done too vigorously, the retina may detach and be discarded along with the vitreous and lens (*see* **Note 14**).
5. Using a pair of forceps, gently detach the retina. Starting at the periphery of the retina, gently scrape the retina off the retinal pigmented epithelium toward the optic nerve. Once the retina is gathered up around the optic nerve, grasp the retina at the optic nerve with the forceps, detach by twisting the forceps a quarter turn, and place detached retina into an amber pill bottle on ice (*see* **Note 15**).
6. Rod outer segments can be isolated immediately or the amber vials containing retinas can be wrapped in two layers of aluminum foil and stored at −80 °C until needed.

3.2 Preparation of Rod Outer Segments from the Retinal Tissue

This purification is based upon the procedures developed by the Papermaster laboratory [2] and relies upon a strategy employing flotation of ROS on high-density sucrose solutions and sedimentation under low-density sucrose solutions to selectively float or pellet crude ROS membranes, to affect their crude isolation from cellular debris. This is then followed by further purification on a discontinuous sucrose gradient to remove other membrane components. All centrifugations are performed in a darkroom at 4 °C.

1. Thaw 100 frozen bovine retinas by floating the bottles in 20 °C water (or one may begin with freshly dissected retina).
2. Transfer thawed retinas to a 250 ml graduated bottle and add an equivalent volume of ice-cold 45 % sucrose solution. There should be at least 50 ml of "empty" volume in the bottle to allow for efficient disruption of the retina in the following step.
3. Seal the graduated bottle with the lid and further seal with Parafilm to avoid leaks during the shaking step and shake vigorously (as hard as you can) by hand for 1 min to disrupt the retinal tissue, shearing off the rod outer segments at the connecting cilium.
4. Distribute the suspension to six 50 ml centrifuge tubes and centrifuge in JA-17 rotor for 5 min at 5,000 rpm (~3,400 × *g*).
5. Pour supernatant from each tube through a gauze-lined funnel into a 250 ml graduated cylinder and dilute the filtrate 1:1 with ice-cold Kühn's buffer. Mix suspension gently by wrapping the end of the graduated cylinder with Parafilm and rocking back and forth slowly by hand. Transfer this diluted supernatant to 50 ml centrifuge tubes and centrifuge in a JA-17 rotor for 10 min at 10,000 rpm (13,800 × *g*).

6. During this centrifugation step, prepare six 50 ml tubes containing a three-step sucrose gradient (1.11, 1.13, and 1.15 densities), using a cannula attached to a 20 ml syringe (*see* **Notes 2** and **16**). The preparation of the gradients should be accomplished outside of the darkroom as it is difficult to observe the formation and/or disruption of the interface between the various densities of sucrose solution under darkroom illumination (*see* **Note 16** for additional detail on preparation of sucrose gradients). Fill all tubes with 10 ml of 1.11 solution; next slowly inject 15 ml of 1.13 solution underneath the 1.11 layer, avoiding air bubbles and disturbing the interface between the two solutions. The final layer is formed by injecting an additional 10 ml of 1.15 solution underneath the 1.13 layer. Ensure that all gradients fill the tubes to the same level as the crude ROS resuspension will be layered on the top of the gradient in the dark (Fig. 2a) and it is difficult to discern between crude ROS and the foam that will form during the resuspension of the crude ROS.

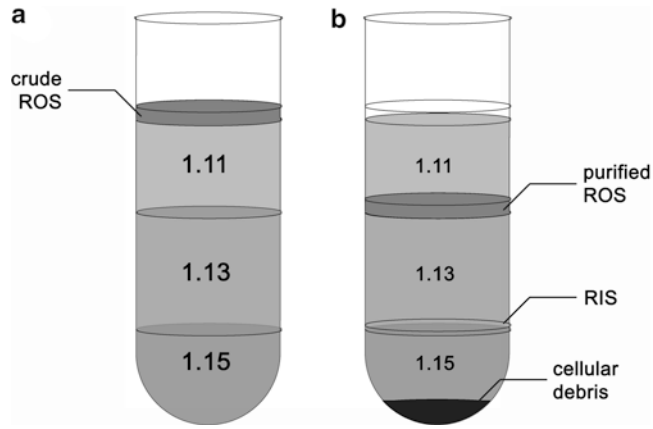

Fig. 2 Sucrose gradient construction and purification of crude ROS. (**a**) Representative diagram of the locations of each density of sucrose solution. Note that construction of the gradient is done in the reverse order, outside of the darkroom; 10 ml of 1.11 density sucrose solution is added first, followed by 15 ml of 1.13 density sucrose solution which is very slowly injected *under* the 1.11 layer; this is followed by the injection of 10 ml of 1.15 density solution *under* the 1.13 layer. These gradients are transported carefully back into the darkroom and the resuspended ROS membranes from Subheading 3.2, and **steps 5** and **7** are layered on top of the 1.11 density solution; for best results, the ROS layer should be very slowly layered on top by allowing the suspension to flow down the side of the tube. (**b**) Location of ROS layer after centrifugation. The ROS layer collects at the interface between the 1.11 and 1.13 density layers. Vesicles derived from the rod inner segment form a weak diffuse band at the 1.13 -- 1.15 interface wheras celular debris collects as a pellet under the 1.15 density solution.

7. After the centrifugation of the ROS suspension, carefully pour off the supernatant and discard. Resuspend the crude ROS membrane pellets in 4–6 ml of 1.10 density solution and 2–3 ml Kühn's buffer using a cannula attached to a 10 ml syringe. Once resuspended, rinse the interior of the centrifuge tubes where the crude ROS pellet was attached with an additional 2 ml of Kühn's buffer to remove any additional membrane pellet stuck to the interior of the tube and combine with the resuspended membranes (*see* **Note 17**).
8. Using a cannula, slowly and gently dispense an equal volume of the resuspended crude ROS pellet down the edge of the tube onto the top of each step gradient. Take care to make sure that the total volume in each tube contains the same volume, as it is difficult to observe the volume of the tubes in the darkroom; allow the layer to settle to observe the interface between the foam formed during the resuspension and the resuspended ROS.
9. Centrifuge for 30 min in a JS-13.1 swinging bucket rotor at 12,000 rpm (22,500 × *g*) with the centrifuge brake turned off (this is critical to preserve the gradient during rotor deceleration *see* **Note 18**).
10. After centrifugation, take great care to not disturb the gradients when removing the centrifuge tubes from the rotor. The ROS membranes will collect at the interface between the 1.11 and 1.13 layers (Fig. 2b). It should be noted that the ROS band is considerably more diffuse when utilizing frozen retina for the purification (*see* **Note 19**).
11. Collect the interface between the 1.11–1.13 layers by gently inserting the tip of the cannula into the ROS band at this interface and gently moving the tip of the cannula around the circumference of the tube while applying suction with the syringe. It is helpful to illuminate the gradient from behind using a flashlight with a red filter while extracting the interface as the ROS membranes appear as an opaque dark band while the gradient solution appears translucent or transparent. Efficiency of removing the ROS layer should be checked after the first tube by removing it to white light and immediately observing if any red layer still exists (indicating incomplete removal of the ROS layer); if there is a red layer still present, then more solution at the interface should be extracted from the remaining gradients.
12. Transfer the purified ROS suspension harvested from this interface to a 250 ml graduated cylinder and diluted 1:1 with ice-cold Kühn's buffer, wrap the end of the cylinder with Parafilm and mix gently by rocking back and forth slowly, and then pellet ROS by centrifugation at 12,000 rpm (22,500 × *g*) for 5 min in the JS 13.1 rotor.

13. Discard the supernatant and leave pelleted ROS. Seal these tubes with Parafilm, wrap with aluminum foil, label, and store at −80 °C until use. Ensure the tubes containing the pellets freeze upright so the ROS pellet does not flow down the side of the tube during freezing (*see* **Note 20**).

3.3 Purification of ROS Membrane Extracts by Size-Exclusion Chromatography

While the ROS preparation removes the majority of soluble proteins present from the retina, resulting in membranes that contain primarily rhodopsin, in many cases it is desirable to further purify the rhodopsin to remove residual membrane proteins. This includes a considerable amount of the apoprotein, opsin, which forms as a result of the cattle/eye harvesting being performed under lighted conditions and its incomplete regeneration back to rhodopsin. Because opsin and rhodopsin chemically differ only in the presence of the covalently bound retinal chromophore, it is difficult to chromatographically separate the two via affinity chromatography. However, when solubilized into detergent solution, opsin is less stable than rhodopsin, allowing for a selective precipitation which employs conditions just harsh enough to precipitate opsin and other membrane proteins while leaving the rhodopsin in solution. In the past, immunoaffinity or anion exchange chromatography has been utilized to further delipidate and purify the rhodopsin after detergent extraction from membranes. We have found that these additional purification steps can be substituted with a single size-exclusion chromatography step on zinc-extracted solubilized membranes. This size-exclusion step enables determination of oligomeric state, estimates of bound detergent amount, and comes with the added bonus of allowing for exchange into a chemically defined buffer and the ability to switch to an alternative detergent (*see* Table 1 and **Note 21**).

3.3.1 Zinc Extraction of Rhodopsin from Purified ROS Membranes

1. Thaw ROS membranes from 100 bovine retinas and resuspend using a 10 ml serological pipette with an electric Pipet-Aid in 4 ml of 50 mM MES, pH 6.3.
2. Quantify the resuspended rhodopsin spectrophotometrically, utilizing the change in the absorbance maximum at 500 nm upon light exposure to calculate the amount of ground-state rhodopsin present (ΔA_{500}). A 1:100 dilution of rhodopsin in the rhodopsin determination buffer is used to blank the spectrophotometer (*see* **Note 22**). After blanking with sample, the sample is removed from the darkroom and exposed to bright light for 5–10 min, and then the absorbance at 500 nm is determined spectrophotometrically (ΔA_{500}). The absolute value of this change in absorbance is proportional to the amount of ground-state rhodopsin in the original suspension, and using the molar extinction coefficient for rhodopsin (40,600 $M^{-1}cm^{-1}$), the molecular weight of rhodopsin (40,000 Da), and the dilution factor (1:100 in this case), the total concentration in mg/ml can be calculated:

$$(100 \times 40{,}000 \times \Delta A_{500} / 40{,}600 = [\text{Rho}]\text{mg/ml}).$$

Typical concentrations of rhodopsin after resuspension are 6–10 mg/ml, depending primarily on the residual buffer left over from the final ROS preparation and the volume of buffer used for resuspension of the pellet.

3. Solubilized the resuspended rhodopsin in a 50 ml polycarbonate centrifuge tube with n-β-D-nonyl-glucoside (NG) at a ratio of 2.2–2.5 mg NG/mg of rhodopsin, which can either be added as a 500 mg/ml stock (in 50 mM MES, pH 6.3) or simply by adding dry powder to the resuspended rhodopsin. Stir the suspension containing detergent and ROS membranes on a magnetic stir plate for 30–60 min at 4 °C to dissolve the detergent and fully solubilize the sample.
4. Measure the volume of sample, and then adjust the concentration of MES to 40 mM with 1 M MES, pH 6.3 stock solution, and enough solid zinc acetate or zinc chloride is added to the suspension to bring the final concentration to 100 mM. Mix the solution on a magnetic stir plate in a 50 ml polycarbonate centrifuge tube for 15–30 min at slow speed and stored on ice overnight (*see* **Note 23**). During this overnight incubation, the resuspended ROS pellet will become opaque due to precipitation of the opsin and membrane lipids.
5. Remove the magnetic stir bar and remove the precipitated opsin, other membrane proteins, and lipids (which have precipitated out of solution due to treatment with zinc) by centrifugation (JA-17 rotor at 10,000 rpm (13,750 × *g*) for 10 min).
6. Carefully decant supernatant and quantify rhodopsin. Rather than measuring the concentration via ΔA_{500} method as described above, a more accurate measure of rhodopsin purity can be obtained by taking the absorbance spectrum of the sample and calculating the ratio of absorbance at 280:500 nm (A_{280}/A_{500}). An A_{280}/A_{500} ratio of 1.56 indicates pure rhodopsin with no other significant contaminating proteins, and at this point in the purification, this ratio should be close to this number. For crystallization of ground-state rhodopsin, the A_{280}/A_{500} ratio should be <1.8.

   Concentration and yield vary based upon total volume into which the ROS membranes were resuspended, but typically from 100 commercially sourced frozen retinas, 60–70 mg of >95 % purity rhodopsin is obtained at a concentration of 5–10 mg/ml. When initiating the purification with the fresh retina, the yield and purity should be slightly higher with 70–80 mg of 98 % pure rhodopsin purified per 100 retinas.

3.3.2 Dialysis to Remove Zinc, Detergent Exchange

While the zinc extraction step is adequate to remove opsin and other membrane proteins, the presence of high concentrations of zinc after extraction may destabilize the rhodopsin in solution or

interfere with downstream assays or further purification steps. It is critical to remove zinc prior to size-exclusion chromatography or concentration in a centrifugal concentrator as zinc spontaneously forms insoluble precipitates of $Zn(OH)_2$ which can irreversibly clog a size-exclusion column or centrifugal concentrator. In some cases it is desirable to replace the high CMC detergent (NG) with a lower CMC detergent such as (DDM) or lauryl maltose neopentyl glycol (LMNG). Dialysis against LMNG, and in some cases even DDM, can result in the precipitation of some or all of the rhodopsin due to the high CMC detergent (NG), dialyzing away before the low CMC detergent can make its way into the dialyzer. To avoid this, it is recommended that dialysis be performed in the presence of 6.5 mM NG to remove excess zinc, which will avoid this result (*see also* **Note 8** for an alternative to dialysis against NG). The use of 1× the CMC of the detergent at this step minimizes the concentration of detergent along with rhodopsin, which when over-concentrated will result in an extremely viscous solution that is difficult to pipette accurately and in some cases may result in protein precipitation/sample loss.

1. Transfer 5 ml of the zinc-extracted rhodopsin to a 5 ml 20 kDa MWCO Float-A-Lyzer/Slide-A-Lyzer and dialyzed against 100 ml of dialysis buffer (6.5 mM NG, 20 mM MES, pH 6.3, 200 mM NaCl, 10 mM EDTA, 1 mM DTT) for 2–4 h at 4 °C.
2. The dialysate is discarded and the dialysis is repeated against an additional 100 ml of buffer for an additional 2–4 h or overnight.

3.3.3 Size-Exclusion Chromatography

1. If the sample is to be further purified and/or detergent exchanged by size-exclusion chromatography, transfer the dialyzed rhodopsin to an Amicon ultra 4 or ultra 15 centrifugal concentrator (MWCO 30 kDa) and concentrate to the desired loading volume for the selected size-exclusion column.
2. The loading volume for a size-exclusion column should not exceed 1–2 % of the total column volume. For example, for a large-scale purification (as described here) when exchanging the rhodopsin into LMNG, 1.25 ml of concentrated rhodopsin is loaded at 20 cm/h onto a Superdex 200 16/600 preparative size-exclusion column which has been pre-equilibrated with size-exclusion buffer (*see* **Note 24**). Figure 3 shows a representative chromatogram of rhodopsin purified in NG and dialyzed against NG containing buffer before being buffer exchanged into LMNG.
3. Following size-exclusion chromatography, measure the A_{280}/A_{500} for all peak fractions and pool fractions containing both a concentration of rhodopsin >1 mg/ml and an A_{280}/A_{500} ratio <1.8. Then concentrate these fractions in an Amicon ultra 4 centrifugal concentrator (MWCO 30 kDa) to desired volume/concentration.

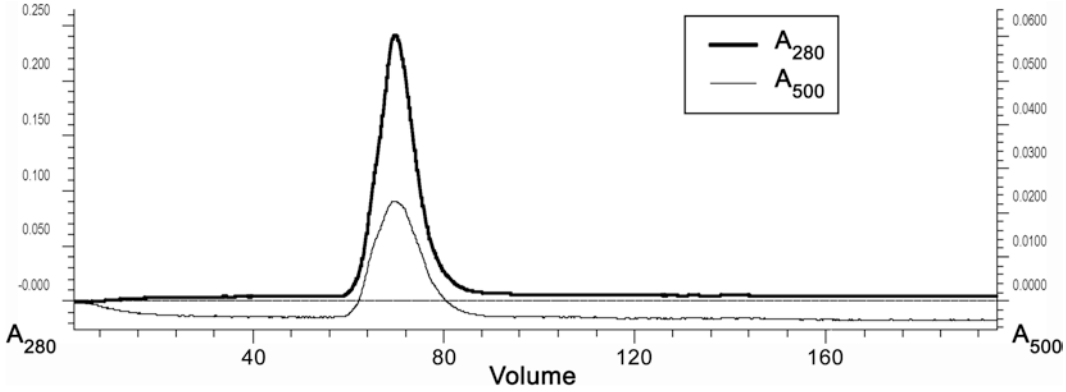

Fig. 3 Representative chromatogram showing separation of zinc-extracted rhodopsin. After dialysis against dialysis buffer consisting of 6.5 mM NG, 20 mM MES, pH 6.3, 200 mM NaCl, 1 mM DTT, and 10 mM Na(EDTA), 10 mg of rhodopsin at a concentration of 10 mg/ml and (1 ml total) of this sample was loaded onto an XK 16/1000 column containing 200 ml of Superdex 200 preparative media which was pre-equilibrated with size-exclusion buffer (20 mM MES, pH 6.3, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, and 2 mM OGNG detergent). Absorbance at 280 nm is plotted as a thick line and absorbance at 500 nm is denoted as a thin line. It is not practical nor is it useful to measure the A_{500} inline when purifying ground-state rhodopsin as this will partially or fully photoactivate the rhodopsin, resulting a significant amount of contaminating opsin. Rather, aliquots of peak fractions should be diluted 1:50 in the rhodopsin determination buffer (10 mM DDM, 20 mM Bis–Tris propane, pH 7.5, and 20 mM hydroxylamine) and their absorbance spectra measured. Samples with A_{280}/A_{500} ratios less than 1.8 and concentrations of rhodopsin greater than 1 mg/ml are then pooled and centrifugally concentrated

4. At this point rhodopsin can be aliquoted into microcentrifuge tubes, wrapped in two layers of aluminum foil, snap frozen in liquid nitrogen, and stored at −80 °C until needed. Alternatively, because of its stability, for short-term storage, rhodopsin can simply be stored protected from light on ice.
5. When exchanged into LMNG or OGNG detergent, rhodopsin can be concentrated to >50 mg/ml with no appreciable increase in viscosity or precipitation. In glucoside/maltoside detergents, concentrations >20 mg/ml, while possible, are discouraged as consequent concentration of detergent micelles along with the rhodopsin during centrifugal concentration results in a viscous solution which is difficult to accurately pipette and manipulate.

4 Notes

1. Eyes are generally discarded by a slaughterhouse along with the brain and bones of the cow/steer, so the price paid to the slaughterhouse is in actuality for the additional labor in removing the eye from the skull. Consultation with the person performing the enucleation is useful as it should be stressed that the eyes should be stored in a lighttight black plastic

bag on ice as soon as they are extracted from the bovine carcass, to avoid additional photoactivation of the rhodopsin. Dissection of the retina should be performed as soon as possible after death as the retina will become less attached to the retinal pigmented epithelium, complicating its intact dissection.

2. If rhodopsin from 200 to 300 retinas is needed, the protocol can be modified to utilize a larger rotor such as a JA-14 for the initial crude isolation of ROS membranes; however, for best results, the resultant crude ROS should be split into two sucrose gradient purification steps.
3. When scaling down the size of the preparation to around 25 retina, it is possible to use a single continuous gradient of sucrose spanning the density range of 1.10–1.15. With fresh, unfrozen retina this should give an extremely tight band for the ROS. Given the time and issues with reproducibility in the preparation of six large continuous gradients, the step gradient methodology detailed here is a reasonable compromise between volume of the ROS layer, purity, and time required for purification.
4. All buffers for the ROS isolation are prepared from Kühn's buffer: preparation of 5 l of Kühn's buffer is more sufficient to prepare the sucrose solutions and perform several ROS isolations. Buffer is prepared from dry ingredients with no adjustment of final pH as indicated in Table 2.

   Prepare 45 % sucrose solution: dissolve 900 g of sucrose in Kühn's buffer to a final volume of 2 l (45 % (w/v)).

   Prepare sucrose gradient solutions: utilizing the 45 % sucrose solution and the Kühn's buffer prepared above, four additional sucrose solutions of defined density are prepared; approximate volumes of each solution required for a single purification and amounts of 45 % sucrose solution and Kühn's buffer required to prepare each solution are listed in Table 3. Pour appropriate volumes of each solution into a 500 ml graduated cylinder. Final specific gravity must be adjusted at 25 °C using a hydrometer.

Table 2
Preparation of Kühn's buffer

Component	Grams (for 5 l)
K_2HPO_4	35.70
KH_2PO_4	17.70
$Mg(C_2H_3O_2)_2 \cdot 4H_2O$	1.072
EDTA-$Na_2 \cdot 2H_2O$	0.186
Dithiothreitol (DTT)	0.771
Sodium azide (optional)	2.5

Table 3
Preparation of sucrose solutions for ROS isolation

Density	Approximate volume of 45 % sucrose (ml)	Approximate volume of Kühn's buffer (ml)	Approximate volume required for 100 retina (ml)
1.15	420	82	60
1.13	330	144	90
1.11	315	208	60
1.10	107	93	6
The following two solutions are also needed for the purification			
45 % sucrose	800 (left over from gradient solution preparation)		100
Kühn's buffer		1,000 (left over from gradient solution preparation)	300

If buffer is intended for use over the course of several weeks to months, DTT should be omitted from initial preparation and added just before use due to its instability in aqueous solutions. Addition of dry DTT to the sucrose solutions avoids changes in the density of the sucrose solution were the DTT stock solution to be added. If sodium azide is included in the solution, storage for longer than 6 months at 4 °C is possible, but solutions should be checked for signs of bacterial/fungal growth prior to use (often manifested as small cotton ball-like growths in the bottom of the bottle).

5. Addition of hydroxylamine to the buffer is only necessary for the ΔA_{500} assay to ensure complete removal of chromophore through formation of the retinal oxime after photoactivation. Hydroxylamine can be included in the dilution buffer for the A_{500} determination of purified rhodopsin with no negative consequences.
6. $ZnCl_2$ can be substituted for the acetate salt in this case. Solid zinc is preferred over zinc stock solutions to minimize generation of the insoluble $Zn(OH)_2$ precipitate.
7. Most alkyl glucoside/maltoside detergents can be used as long as enough detergent is employed to solubilize the entirety of the membranes and the rhodopsin; OG, NG, and DDM have all been used successfully for the solubilization and zinc extraction of rhodopsin. Use of a high CMC detergent such as OG or NG enables detergent removal/exchange during dialysis.
8. Detergent exchange can be accomplished during the removal of zinc, but dialysis against low CMC detergents must be preceded

by a preincubation of the sample with adequate amounts of the desired detergent, such that the concentration of new detergent is isotonic with the dialysate; if this preincubation is neglected, the solubilizing detergent dialyzes away from the rhodopsin more rapidly than the low CMC detergent can dialyze into the dialyzer, resulting in widespread precipitation of the rhodopsin.

9. Alternatively, standard dialysis tubing and clips can be used, although the cartridge type dialyzers are much easier to load/unload in the darkroom. Dialysate should be stirred during dialysis.
10. The red filter material can be obtained from a photographic or theatrical supply store. This film can be used to convert a white light to a red safelight. It is also available as tubes that fit over standard fluorescent light bulbs. This film should be replaced on a regular basis as it begins to lose its efficiency over time, resulting in a more orange transmission spectrum.
11. Low CMC detergents are considerably more cost-effective as significantly less detergent is required for purification.
12. Keep in mind that there is a possibility of exposure to prions in handling bovine eyes as they are in fact neurological material, which could result in the contraction of bovine spongiform encephalopathy ("mad cow disease"). Proper protective equipment should be worn during all steps of the procedure; this should include at minimum: gloves, goggles, and a lab coat. Institutional rules for disposal of neurological material must be followed for the disposal of eye tissue.
13. Some people prefer to use a fresh single-edged razor blade to make the initial incision in the eye and then use surgical scissors to complete the incision.
14. Premature detachment of the retina at this step can also be caused by failing to store the intact eyes on ice immediately after death.
15. The retina is quite delicate and has the structural integrity of wet tissue paper. Great care should be taken to only gently tease the retina off from the RPE layer; avoid overzealous scraping of the eyecup as significant amounts of RPE and other tissue will contaminate the retina, resulting in a poorer-quality final preparation of ROS/rhodopsin.
16. When preparing sucrose step gradients with the sucrose stock solutions, it is advantageous to have adequate lighting (i.e., do not attempt to prepare in the darkroom) and the tubes at eye level in a tube rack that securely holds the tubes, thus ensuring that one can observe the higher-density solution filling the space under the lower-density solution. For best results, flow of the higher-density solution must be slow enough that no mixing with the adjacent layer occurs.

17. It is advantageous to use new centrifuge tubes for this step as scratches and discolorations found on used tubes can be mistaken for residual ROS membranes. Under the dim red light illumination of a darkroom, it is difficult to determine if all the ROS pellet has been resuspended; after removal of the resuspended ROS, the tube can be inspected using a flashlight with red film over the bulb.
18. Modern centrifuges have considerably less internal friction than the older J-2/J-20 centrifuges with which these procedures were developed. Turning the brakes off with a new centrifuge may result in a deceleration that takes more than 2 h! In newer centrifuges there are gentle braking options which can be employed to slow the rotor without disturbing the gradients in a reasonable (~10 min) time frame.
19. When harvesting the ROS layer, it is sometimes necessary to take the entire 1.11 and the majority of the 1.13 layer. This is not a major issue, but may require a second "pelleting" run to harvest the ROS from the solution. Nevertheless, the total amount of gradient harvested should be minimized, as while the A_{280}/A_{500} ratio may not appreciably change when additional gradient solution is harvested, additional nonprotein components such as additional lipids sediment into the 1.11 and 1.13 layers leading to their co-purification.
20. Optionally, the pelleted ROS membranes from the above step after centrifugation can be resuspended in ~30 ml of Kühn's buffer and pelleted in a single tube for storage purposes.
21. The amount of detergent required for solubilization of ROS membranes is considerably higher than would be desired for a crystallization experiment, and the actual free concentration of detergent is unknown due to the inability to control for the amount of lipid that forms mixed micelles during solubilization, the amount of detergent that binds the rhodopsin, and the consequent loss of detergent during the zinc extraction process.
22. Spectrophotometer should be located in the darkroom for ease of measurement if possible. Otherwise, all dilutions for rhodopsin concentration and purity assay must be prepared in the darkroom, wrapped in foil, and read immediately on the spectrophotometer. A dark cloth can be used to assist in the protection of the sample from light.
23. Alternatively, a 2–4-h room-temperature zinc extraction can be performed; if this is extended further, some of the rhodopsin will also precipitate. When performing this extraction, after centrifugation the pellet will be pale pink; a red pellet indicates the precipitation of rhodopsin along with opsin, other membrane proteins, and lipids.
24. In the case of expensive detergents, the column can be equilibrated 60% prior to beginning chromatographic separation, as

the column will be fully equilibrated before the rhodopsin peak elutes from the column. While the column can be completely equilibrated prior to the start, this minimizes the amount of detergent required.

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