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INVESTIGATING THE NANODOMAIN ORGANIZATION OF RHODOPSIN IN NATIVE MEMBRANES BY ATOMIC FORCE MICROSCOPY

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Abstract

Membrane proteins play an integral role in cellular communication. They are often organized within the crowded cell membrane into nanoscale domains (i.e., nanodomains), which facilitates their function in cell signaling processes. The visualization of membrane proteins and nanodomains within biological membranes under physiological conditions presents a challenge and is not possible using conventional microscopy methods. Atomic force microscopy (AFM) provides an opportunity to study the organization of membrane proteins within biological membranes with sub-nanometer resolution. An example of a membrane protein organized into nanodomains is rhodopsin. Rhodopsin is expressed in photoreceptor cells of the retina and upon photoactivation initiates a series of biochemical reactions called phototransduction, which represents the first steps of vision. AFM has provided an opportunity to directly visualize the packing of rhodopsin in native retinal membranes and the quantitative analysis of AFM images is beginning to reveal insights about the nanodomain organization of rhodopsin in the membrane. In this report, we outline procedures for imaging rhodopsin nanodomains by AFM and the quantitative analysis of those AFM images.

Keywords

Atomic force microscopy; biological membrane; membrane nanodomains; membrane protein; membrane structure; photoreceptor cell; receptor oligomerization

1. Introduction

Membrane proteins are estimated to account for about 25% of the human proteome (1). Membrane proteins are embedded in lipid bilayers and play key roles in many critical cellular functions. Due to their abundance and diverse functional activities, dysfunction in these proteins plays a large role in human disease and therefore about 60% of drug targets are membrane proteins (2,3). Membrane proteins are often organized into nanoscale domains (i.e., nanodomains) within biological membranes that are important for the signaling processes that they mediate (4–7). The small sizes of these domains, in the range

of 10–200 nm, has hindered their characterization by conventional microscopy methods (8). Advances in technology have resulted in newer microscopy methods with adequate resolution to visualize and detect these nanodomains that play important roles in cellular signaling (9).

Atomic force microscopy (AFM) is an imaging tool uniquely suited to study the membrane organization and structures of membrane proteins (10,11). AFM can achieve sub-nanometer lateral and vertical resolution under physiological conditions (10), which is sufficient to resolve individual protein molecules and more than adequate to resolve nanodomains in biological membranes. In contrast to high-resolution light microscopy-based methods, AFM does not require membrane proteins to be tagged or modified, and therefore membrane proteins can be investigated in the native membranes in which they are found in nature.

Rhodopsin is a membrane protein belonging to the G protein-coupled receptor (GPCR) superfamily of proteins. It is found in rod photoreceptor cells of the retina and serves as the initiator of vision by absorbing photons of light (12). Rod photoreceptor cells are highly compartmentalized. Rhodopsin is present in the rod outer segment (ROS) of rod photoreceptor cells (Fig. 1A). The ROS consists of 500–2000 stacked discs that are encased by a plasma membrane (13). The discs are double lamellar membranes linked together by a rim region. Rhodopsin is unique among most GPCRs and membranes proteins in that it is embedded in the lamellar region of disc membranes at high concentrations. Rhodopsin is packed at an average density of about $20,000 \mu\text{m}^{-2}$ and occupies about 30% of the surface area of the lamellar region of discs (14). Rhodopsin is the predominant protein species in the ROS disc membranes, accounting for about 90% of the protein content (15). The ability to isolate ROS disc membranes from the retina of vertebrate species and the high concentrations of the receptor in the membrane with limited contamination of other proteins have allowed for the investigation of rhodopsin membrane organization by AFM.

AFM provided the first high-resolution view into the membrane organization of rhodopsin in native ROS disc membranes. The first published AFM image of rhodopsin organization in disc membranes revealed densely packed para-crystalline arrays of dimeric rhodopsin molecules (16). But all the follow-up studies have consistently shown a nanodomain organization of rhodopsin and this organization appears to be conserved among vertebrates (14,17–20). These nanodomain structures consist of dimeric rhodopsin arrays similar to those observed in the first AFM image (20). Dimeric rhodopsin arrays forming nanodomains in disc membranes have also been observed by cryo-electron tomography of the ROS (21). The exquisite sensitivity of rod photoreceptor cells allows for the detection of single photons (22). The supramolecular organization of rhodopsin in disc membranes in the form of nanodomains can provide the necessary platform to achieve the signaling efficiency required for single photon detection within a crowded membrane environment (23,24,21).

Quantitative analysis of AFM images of single ROS disc membranes is revealing novel insights about the structure of discs and factors contributing to the packing of rhodopsin in nanodomains. The ability to examine individual ROS disc membranes has revealed heterogeneity in the size, number and density of rhodopsin nanodomains among individual ROS discs. Examining this heterogeneity can provide important insights into how rhodopsin

organization can be modulated by the cell. For instance, correlation analysis of ROS disc membrane properties led to the idea that the photoreceptor cell adapts to maintain a constant concentration of rhodopsin in the membrane (17), which was later tested and supported experimentally in mice expressing only half the complement of rhodopsin (14). Since nanodomains are comprised of oligomeric arrays of rhodopsin (20), the size of nanodomains reflects the size of rhodopsin oligomers. Histogram and correlation analyses have indicated that the oligomerization of rhodopsin is a concentration-dependent process where oligomeric size increases with receptor concentration (17,14). The concentration-dependent oligomerization of rhodopsin is also supported by quantitative Förster resonance energy transfer studies (25).

These studies have laid the groundwork for future studies that will provide much needed insights into the functional role and determinants of rhodopsin supramolecular organization within ROS disc membranes. These insights will advance our molecular understanding about phototransduction, photoreceptor cell biology, and retinal disease. In the following sections, we describe the methods for imaging ROS disc membranes by AFM and quantitatively analyzing AFM images to extract information about ROS disc membrane structure and rhodopsin nanodomains.

2. Materials

1. Retina to prepare ROS disc membranes
2. Hand-held pestle
3. OptiPrep (Sigma-Aldrich, St. Louis, MO, USA)
4. Ringer's buffer: 10 mM 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (HEPES), 130 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl₂, 1.2 mM CaCl₂, 0.02 mM EDTA, pH 7.4
5. Buffer A: 2 mM Tris-HCl, pH 7.4
6. Buffer B: 2 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 7.4
7. Imaging buffer: 20 mM Tris, 150 mM KCl, 25 mM MgCl₂, pH 7.8
8. Round mica substrates with a diameter of about 1 cm. Examples of preparing mica substrates can be found in previous reports (26,10).
9. Scotch tape
10. Atomic force microscope. The Multimode AFM (Bruker Corporation, Santa Barbara, CA, USA) and Keysight 5500 AFM (Keysight Technologies, Santa Rosa, CA, USA) have been used in our studies. Atomic force microscopes that are equipped with red or infrared laser will not appreciably bleach rhodopsin (27).
11. Si₃N₄ AFM tip. For contact mode and tapping mode, silicon nitride tips (DNP-S, Bruker Corporation, Camarillo, CA, USA) with nominal spring constants of 0.06 N/m and 0.24 N/m, respectively, have been used (see Note 1).

12. SPIP (Image Metrology A/S, Hørsholm, Denmark) image processing software

3. Methods

Rhodopsin absorbs light with a maximal absorbance at around 500 nm and therefore all procedures need to be conducted in the dark under dim red light conditions to avoid bleaching. Existing light sources can be covered with red filters.

3.1. ROS disc membrane preparation (see Note 2)

An overview of the ROS disc membrane preparation is provided in Fig. 1.

1. Dark adapt 10–15 mice by placing mice in the dark overnight. Euthanize dark-adapted mice by CO₂ exposure (see Note 3).
2. Carefully remove the eyes from the mice and isolate the retina (Fig. 1A). Place the retinas in a 1.5 mL microcentrifuge tube containing 300 µL of 8% (v/v) OptiPrep in Ringer's buffer (see Note 4).
3. Prepare a 10–30% (v/v) continuous gradient of OptiPrep in 12 mL Ringer's buffer in a high-speed centrifuge tube (see Note 5).
4. Vortex the 8% OptiPrep solution containing the retinas at maximum speed for 1 min (see Note 6). This step mechanically separates the ROS from the rest of the rod photoreceptor cell.
5. Centrifuge the solution at $238 \times g$ for 1 min at 4 °C (see Note 6). Collect the supernatant, which contains the ROS, and carefully add it to the top of the 10–30% OptiPrep gradient without disturbing the gradient.
6. Add 300 µL of 8% OptiPrep in Ringer's buffer to the pellet.
7. Repeat steps 4–6 five more times.
8. Centrifuge the gradient solution at $26500 \times g$ for 50 min at 4 °C with the deceleration set at zero.
9. A dense band should be present two-thirds of the distance from the top of the gradient. This band contains the purified ROS (Fig. 1B) and should be carefully collected in a high-speed centrifuge tube.
10. Dilute the collected material at least 4-fold with Ringer's buffer.
11. Centrifuge the diluted material at $627 \times g$ for 3 min at 4 °C.
12. Collect the supernatant and centrifuge it at $26500 \times g$ for 30 min at 4 °C. Discard the supernatant and resuspend the pellet in 1 mL buffer A. Transfer the suspension into a 1.5 mL microcentrifuge tube and incubate overnight at 4 °C to osmotically burst the intact ROS (Fig. 1C).
13. Centrifuge the suspension at $16100 \times g$ for 5 min at 4 °C. Discard the supernatant and resuspend the pellet in 25 µL buffer A and homogenize using a hand-held pestle. Add 1 mL of buffer A and mix the suspension. Repeat this step two more times.

14. Repeat step 13 with buffer B (see Note 7).
15. Centrifuge the suspension at $16100 \times g$ for 5 min at 4 °C. Discard the supernatant and resuspend the pellet in 25 μ L Ringer's buffer. Homogenize using a hand-held pestle. Store the final ROS disc membrane preparation at 4 °C and cover tube with foil to prevent bleaching of rhodopsin (see Note 8).

3.2. Preparation of samples for AFM imaging

1. Dilute ROS disc membrane samples (0.5–1 mg/mL) 100–200-fold in Ringer's buffer prior to AFM experiments. Samples should be diluted to minimize background debris while still observing sufficient numbers of ROS disc membranes to image (see Note 9).
2. Cleave the mica surface with scotch tape to remove a layer of the mica to expose an atomically flat surface.
3. Add 30 μ L of the diluted ROS disc membrane sample onto freshly cleaved mica. Incubate for 10 min at room temperature.
4. Remove the liquid from the mica surface using a pipette. Rinse the mica surface by adding 30–50 μ L of Ringer's buffer and gently pipetting the buffer up and down. Discard the buffer and repeat the procedure at least four more times.
5. Add imaging buffer onto the mica support and mount the support onto the atomic force microscope using the appropriate fluid cell (see Note 10).

3.3. AFM imaging of ROS disc membranes (see Note 11)

1. Set the atomic force microscope for either contact mode or tapping mode imaging. Set the instrument so that height and deflection images are generated in contact mode and height and amplitude images are generated in tapping mode.
2. Mount the AFM tip onto the holder or scanner, depending on the instrument, and align the laser so that it is focused on the tip of the cantilever and achieves an optimal sum value.
3. Set horizontal and vertical deflection between –0.5 to 0.5 so that the reflected laser is positioned near the center of the photodiode detector.
4. For tapping mode, perform a frequency sweep to identify the appropriate frequency needed to oscillate the cantilever.
5. Set the scanning area to 0 μ m and engage the AFM tip.
6. For contact mode, retract the tip after engagement by lowering the set point. Set the scan area to the maximum. Gradually extend the tip by increasing the set point. Once the tip is in contact with the surface, apply sufficient force (100 pN or less) to maintain good contact with the sample surface but not damage or deform the sample.

7. For tapping mode, adjust the drive and set point to get the desired force for imaging (less than 100 pN). Set the scan area to the maximum and begin scanning.
8. Optimize scan rates and gains to enhance image quality. Scan rates of 4–6 Hz have been used for contact mode and 0.85–1.42 Hz for tapping mode.
9. Locate an adsorbed ROS disc membrane and zoom in to a scan frame size of 2–3 μm . Capture a final image of a single ROS disc membrane at a resolution of 512 lines per frame (see Note 12 – 14).

3.4. AFM image analysis (see Note 15 and 16)

The methods outlined here are specifically based on the commercially available image processing software SPIP, which is designed for AFM images. Other imaging processing software that have similar capabilities can also be used.

1. Open the raw image file in SPIP.
2. Zoom in and crop the selected image so that the ROS disc membrane fills the frame.
3. For height images, flatten the image using a first order plane correction and set the background to 0.
4. Use the deflection or amplitude image to determine the number and size of nanodomains, size of the disc membrane and inner disc area (Fig. 2).
5. Number and size of nanodomains (Fig. 2B): Nanodomains can be detected using the 'Particle and Pore Analysis' module using the 'Advanced Threshold' detection method. Nanodomains are detected as particles. Select the area containing the nanodomains using the 'Draw Polygon Area of Interest' function and then use the 'RMS Factor Threshold' mode to semi-automatically detect nanodomains. Two parameters must be set in this mode to detect nanodomains. 'Particle RMS Factor' is a factor used to compute the z-value threshold at which a feature should be classified as a nanodomain. The inputted factor will be multiplied by the root mean square (i.e., standard deviation) of z-values in the selected region of the image to automatically compute the z-value threshold. 'Split Particles' setting splits multiple nanodomains initially detected as a single nanodomain. The split level will be established relative to the previously established z-value threshold by inputting a factor that will be multiplied by the root mean square of z-values. Both parameters must be adjusted manually to obtain the optimal detection of nanodomains. Numerical values in the range of $-1.2 - 0$ and $1.5 - 4$ are typically used for the 'Particle RMS Factor' and 'Split Particles' settings, respectively. The software will automatically compute the number and size of detected nanodomains.
6. Since the nanodomains are formed by oligomeric rhodopsin molecules (20,21), the average area occupied by a rhodopsin molecule within such an oligomeric

complex is 14 nm^2 (20,28). The area of a nanodomain is divided by this number to estimate the number of rhodopsin molecules present in the nanodomain.

7. Disc membrane size (Fig. 2C): The diameter of the disc can be measured using the 'Caliper' tool in the 'Measure Shapes' panel.
8. Inner disc area (Fig. 2D): The area of the ROS disc membrane excluding the rim region can be measured using the 'Polygon Shape' tool in the 'Measure Shapes' panel by highlighting the corresponding area. The computed inner disc area can be used to determine the density of nanodomains and rhodopsin molecules in the ROS disc membrane.
9. The height image is analyzed for determining the height of features in the image (e.g., Fig. 3). To determine the height of nanodomains, they should be detected as outlined in Step 5 and the software will automatically determine the height of the detected nanodomains.

4. Notes

1. Selection of the AFM tip is critical for visualizing rhodopsin nanodomains in ROS disc membranes. Tips should be high quality and sharp in order to image with sufficient resolution to resolve individual nanodomains. We routinely use DNP-S AFM tips (Bruker Corporation, Camarillo, CA, USA). Tips with equivalent or greater sharpness can also be used.
2. ROS disc membranes have been prepared from murine, human, and *Xenopus laevis* retina for AFM studies on rhodopsin nanodomains (29,17,18). The methods outlined here are those for murine retina. Similar methods can be employed for retina from other species. Notes are included when the preparation step deviates from those presented for murine samples.
3. For preparation of *X. laevis* ROS disc membranes, 5–6 frogs are dark-adapted prior to euthanasia in a solution of 0.26% tricaine. Since human retina are obtained from donor eyes, dark adaptation is not possible. To ensure minimal bleaching of rhodopsin, donor eyes should be procured quickly (we have utilized eyes procured 3.5–18 h after death) in a darkened room and shipped in a light-tight container within 24–36 h after death. For a single gradient, only a small fraction of the retina from a single donor eye is sufficient.
4. For preparation of *X. laevis* ROS disc membranes, frog Ringer's buffer (3 mM HEPES, 111 mM NaCl, 2.5 mM KCl, 1.6 mM MgCl_2 , 1.0 mM CaCl_2 , and 10.0 mM D-Glucose, pH 7.8) should be used instead of Ringer's buffer in all steps.
5. A 20–40% (v/v) continuous gradient of OptiPrep in 12 mL frog Ringer's buffer should be prepared for *X. laevis* ROS disc membrane preparations.
6. The ROS of *X. laevis* is more fragile compared to murine ROS. Thus, the suspension of retina should be vortexed at half-maximal speed for 15 s. The suspension should then be centrifuged at $100 \times g$ for 30 s.

7. This step should be omitted for *X. laevis* ROS disc membrane preparations in order to minimize fragmentation of ROS discs.
8. Fresh ROS disc membrane samples should be used for AFM. Samples should be used within 2–3 days of preparation. After this time, most of the ROS disc membranes become degraded and mostly debris is adsorbed on the mica surface. ROS disc membranes are unstable at higher temperatures (e.g., 37 °C) (18), and therefore should be stored at 4 °C.
9. A sufficient dilution should result in 1–3 adsorbed ROS disc membranes visible in a 10 $\mu\text{m} \times 10 \mu\text{m}$ scan area.
10. Fluid cells will be different depending on the type of atomic force microscope used. For the Bruker Multimode atomic force microscope, the fluid ports in the fluid cell can be loaded with 300 μL of imaging buffer, which will maintain a constant amount of fluid on the sample and counteract evaporation. For the Keysight 5500 atomic force microscope, the mica support will be placed in a reservoir in the fluid cell itself. The reservoir with sample should be filled with 300 μL of imaging buffer and the buffer should be replenished as needed to counteract evaporation.
11. For general considerations for optimizing conditions for imaging membrane proteins at high resolution, please refer to (10).
12. Almost all of the ROS disc membranes deposited on mica exhibit only a single membrane layer (Figs. 2, 3A, and 3B). These single layered ROS disc membranes adsorb on mica predominantly exposing the extracellular surface (27). Less than 5% of the deposited ROS disc membrane preparation contains intact discs that contain a double membrane (Figs. 3C-3F). The height of a single layer ROS disc membrane is about half of that of an intact disc within the lamellar region (Fig. 3)(18). Regardless of whether the disc is intact or only displays a single membrane, rhodopsin nanodomains are present and can be visualized by AFM. Nanodomains are observed both in membranes in direct contact with the mica substrate and those that are free from the mica substrate.
13. Single layered ROS disc membranes are used for analysis since the nanodomains are clearly defined. These ROS disc membranes display a distinct topography in AFM images with a rim region (label 1, Fig. 2A; label 4, Fig. 3A) and lamellar region (label 2, Fig. 2A; label 3, Fig. 3A). Rhodopsin is excluded from the rim region and is the predominant protein species in the lamellar region (30,27,19). The lamellar region contains a lipid bilayer without protein with a height of 4 nm and rhodopsin embedded in a lipid bilayer in the form of nanodomains with a height of 8 nm (17). The rim region displays variable heights, which are greater than heights exhibited in the lamellar region (Fig. 3A). The rim region in ROS disc membranes should be well-defined with a majority of the rim region still intact around the periphery of the ROS disc membrane. This criterion ensures that there is minimal disruption of the disc membrane structure and that the organization of rhodopsin observed is likely that present in intact photoreceptor cells. Despite ROS disc membranes being somewhat disrupted, the nanodomain organization of rhodopsin observed by AFM appears to be unperturbed and represents the physiological organization, since the results are consistent with those obtained by alternate methods. For instance, estimates of rhodopsin density

derived from alternate methods are consistent with estimates made by AFM and the nanodomain organization has also been observed in intact ROS where discs are not disrupted (14,21).

14. Tapping mode is often preferred for imaging biological samples because it exerts less lateral force on the sample compared to contact mode. Biological samples that are easily disrupted or deformed by applied force should be imaged by tapping mode. However, for biological samples that are sufficiently stable to the applied force by the AFM tip, contact mode is preferred since images can be acquired faster and higher resolution can often be achieved. Both contact mode and tapping mode have been utilized to image ROS disc membranes (Fig. 4). Tapping mode is less disruptive, allowing for better definition and structure of the rim region (Fig. 4B). However, on the whole, images generated by both imaging modes are comparable. Nanodomains of rhodopsin appear similarly in either contact mode or tapping mode and results of quantitative analysis of nanodomains are equivalent (14). Thus, rhodopsin nanodomains can be imaged by either imaging mode. Since up to 100 images or more are required in our studies, contact mode imaging is preferred because of faster acquisition times compared to that in tapping mode imaging.

15. Some of the basic processing of images and analysis of features in the images can be performed using the software that accompanies the atomic force microscope. Our first quantitative study on rhodopsin nanodomains was exclusively performed using software accompanying the Bruker Multimode atomic force microscope (17). The dimensions of ROS disc membranes and sizes of individual nanodomains were manually measured using this software. Nanodomains were assumed to be elliptical in shape, which is not the case when detected semi-automatically (Fig. 2B). Manual analysis is time consuming and therefore not practical for large amounts of data. Moreover, it is less accurate than semi-automated analysis methods based on image processing software such as SPIP (14).

16. We have analyzed 50–100 images obtained from different conditions, which was sufficient to detect differences in comparisons of mean values and correlation analyses (14). The reliability of the results will improve with more data collected; so, we suggest analyzing at least 100 images.

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Figure 1.

Overview of the ROS disc membrane preparation. (A) Retina are isolated from the eye. Shown is a light microscopy image and cartoon of photoreceptor cells composed of rod outer segments (ROS), rod inner segments (RIS), and outer nuclear layer (ONL). Scale bar, 15 μm . (B) The ROS is isolated and purified. Shown is a cartoon and light microscopy image of purified ROS. Scale bar, 15 μm . (C) ROS discs are obtained by osmotically bursting the ROS. The resulting ROS disc membranes are adsorbed onto a mica substrate and imaged by AFM. This figure is reproduced from (17) with permission from Elsevier.

Figure 2. Overview of the analysis of ROS disc membrane images using SPIP. Scale bar, 500 nm. This figure is reproduced from (14) with permission from the American Chemical Society.

Figure 3.

(A, B) Height (A) and deflection (B) image of a single layered ROS disc membrane. Height profiles are shown from line scans highlighted in the height image. Features are labeled as follows: 1, mica; 2, lipid bilayer without protein; 3, lamellar region; 4, rim region. Scale bar, 500 nm. (C-G) Height (C, E) and deflection (D, F) images of an intact ROS disc imaged at low and high forces. A height profile is shown from a line scan highlighted in panel E (G). Features are labeled as follows: 1, rim region; 2, lamellar region. Scale bar, 250 nm. The figures are reproduced from (17), with permission from Elsevier, and from (18).

Figure 4. (A) Deflection images of ROS disc membranes obtained by contact mode. (B) Amplitude images of ROS disc membranes obtained by tapping mode. Scale bar, 500 nm. The figures are reproduced from (17), with permission from Elsevier, and from (14), with permission from the American Chemical Society.