

Biomimetic Sensing by the Photoreceptor Membrane Protein Bacteriorhodopsin

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Bacteriorhodopsin (bR) is a light-sensitive protein which has a proton-pump function, and is found in the cell membrane of *Halobacterium salinarum* inhabiting the salt lake. Optical excitation of bR at an electrode-electrolyte interface generates differential photocurrents while an incident light is turned on and off. This unique functional response is similar to that seen in retinal neurons. This paper describes the fabrication and evaluation of the dip-coated bR thin films, photocurrent characteristics, and biomimetic artificial retinas with the bR thin films.

1 Introduction

Research has been carried out in recent years in multiple fields to learn about the superior functions of organisms. Organisms created by nature over several billion years possess nano-scale, high performance biomolecules that are difficult or impossible to mimic with current technology. It is thought that we will be able to create new biomimetic artificial systems that make use of the high functionality of biological systems by fusing these biomolecules with existing technology. In order to achieve this at NICT, we aim to create an environmentally-friendly, low cost, original ICT device industry and a communication paradigm through research and development. We carry out research and development into biomimetic optical sensing using bacteriorhodopsin, a photoreceptor membrane protein extracted from the cell membrane of *Halobacterium salinarum* that lives in saline lakes.

Halobacterium salinarum is an archaebacterium that thrives in saline lakes, salt pans and other hypersaline environments. It proliferates in saline lakes such as Lake Retba in Senegal, Africa, where it is known to make the water purple. In 1971, D. Oesterhelt of the Max Planck Institute of Biochemistry and W. Stoeckenius of the University of California proved that this purple color comes from a bacterium known as *Halobacterium salinarum* which lives in hypersaline environments^[1]. *Halobacterium salinarum* is the scientific name of the bacterium (common name: *Halobacterium halobium*); its cell membrane contains a purple membrane protein. Like

rhodopsin, the visual pigment of animals, the constituent of this protein is retinal chromophore (vitamin A aldehyde), and because it is rhodopsin found in bacteria, it is called bacteriorhodopsin (bR). Its function is also similar to that of rhodopsin, and it has a light-driven proton pumping function^[2]. The photoreaction of bR, like rhodopsin, begins with the photoisomerization of the retinal, but unlike rhodopsin, which is metabolized through photolysis, it returns to its original state as seen in the photochemical reaction cycle shown in Fig. 1. The letters in the diagram represent intermediates, while the subscripts show the maximum absorption wavelength. When bR in the ground state absorbs yellow light it passes through various intermediates to enter a metastable state and becomes an M intermediate (bR \rightarrow J \rightarrow K \rightarrow L \rightarrow M),

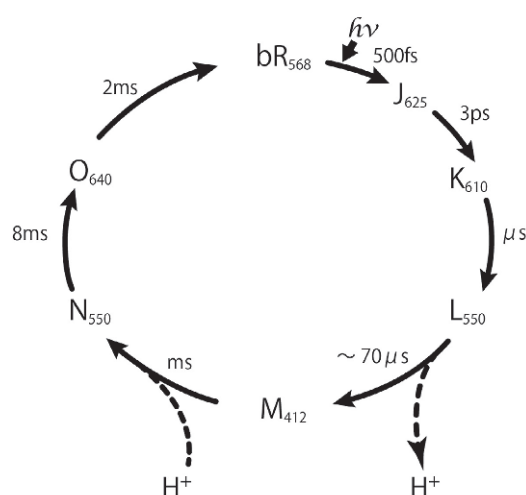


Fig. 1 Photochemical reaction cycle of bacteriorhodopsin (bR)

and a retinal proton (H^+) is discharged from the cell. The M intermediate returns to its ground state through thermal relaxation ($M \rightarrow N \rightarrow O \rightarrow bR$) and the proton is reabsorbed in this process. The series of photochemical reactions in this cycle take place over approximately 10 milliseconds at room temperature^{[3][4]}. *Halobacterium salinarum* makes use of this proton pumping function to create a proton concentration gradient across its cell membrane, and it generates energy through its ATP synthase system driven by the difference in electrochemical potential^[5]. In this way, bR differs from the visual pigment of animals in that it is not metabolized through photoirradiation, but it is a stable photoreceptor membrane protein that can undergo repetitive photochemical reactions. It is ideal for use in applied engineering research because of its high stability in photochemical reactions and heat stability compared to other proteins. Attempts to apply bR in photoelectric response devices, nonlinear optical response devices, real-time hologram recording, spatial modulators, etc., have been reported^{[6]-[11]}. Irradiation of bacteriorhodopsin on the interface of electrodes and the electrolyte generates a time-differential photocurrent, which in turn generates a signal that responds only to changes in the intensity of light without the need for an external power supply. A characteristic of this time-differential photocurrent is its similarity to encoded optical information by the retina of humans and other animals making bR a useful biomaterial in biomimetic optical information processing, such as visual functions. In this paper we will report on the fabrication and evaluation of bR thin films using the dip coating method suitable for biomolecules, the time response characteristics of bR photoresponse cells, and the creation of a bipolar bR photosensor which simulates the visual receptive field of visual functions.

2 Cultivating and refining bacteriorhodopsin (bR)

The biomaterial bR is a resource with little environmental burden which is made by cultivating and refining *Halobacterium salinarum* in the laboratory. *Halobacterium salinarum* is adapted to environments with extremely high concentrations of salt such as saline lakes and salt pans, and it thrives best in water of 4 M (mol/L), which corresponds to near-saturation with salt. Other bacteria cannot survive under such extreme conditions, so it is cultivated easily in the laboratory. This halobacterium

is a long, thin, rod-shaped cell that is from a few μm up to around 10 μm in length, as shown in Fig. 2, and it has several flagella on both ends of the cell. Its cell walls are made of proteins and sugars, with an inner cell membrane. The cell membrane consists of a purple membrane and red membrane, and the purple membrane is disc-shaped with a diameter of around 0.5 μm and a thickness of around 4 nm. The purple membrane is made of bR protein, while the red membrane is made of red pigment known as bacterioruberin, which blocks near ultraviolet radiation. We obtained wild strains of *Halobacterium salinarum* (provided by RIKEN BioResource Center and the Kubo Lab. of Soka University) that create large quantities of bR purple membrane, cultivated them, and refined their bR. Figure 3 shows the growth curve of bacteria during cultivation. As the result of measuring the changes in absorbance (at the wavelength of 560 nm) of a culture medium with a spectrophotometer, it can be seen that the medium becomes saturated with *Halobacterium salinarum*

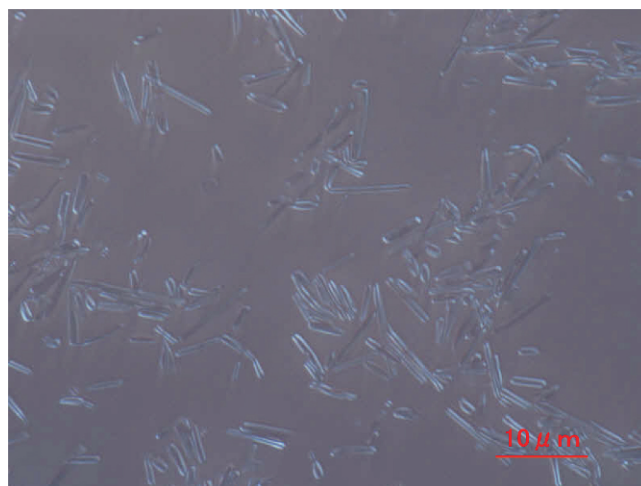


Fig. 2 *Halobacterium salinarum*

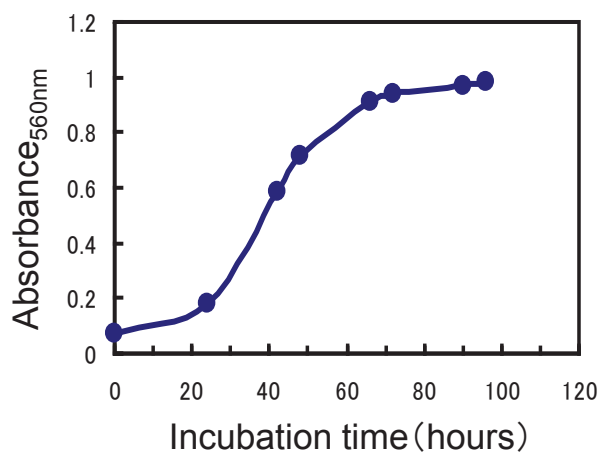


Fig. 3 Growth curve of *Halobacterium salinarum*

after approximately four days. The cultivated bacteria were ground, then separated through dialysis, centrifugation, and sucrose density gradient centrifugation to extract the purple membrane^{[12][13]}. Figure 4(a) shows the bR suspension, and (b) shows it in powdered form made by the freeze-drying method. The amount of purple membrane harvested was 13 mg when 1 liter was cultivated, and 240 mg when 10 liters were cultivated. Figure 5 shows the optical absorption characteristics of the refined bR purple membrane and red membrane. As mentioned above, as the bR purple membrane releases a proton from the ground state in the photochemical reaction cycle to enter a metastable state, it has an absorption maximum at a wavelength of around 570 nm.

3 Fabricating and evaluating bR thin films by the dip coating method

The cast method, electrophoretic sedimentation technique, Langmuir-Blodgett (LB) technique, layer-by-

layer deposition technique, etc., were traditionally used to immobilize bR to electrodes^{[6][14]-[17]}. We implemented the dip coating method for the first time in fabricating bR thin films^[18]. This method not only allows efficient formation of thin films from the bR refined and extracted from *Halobacterium salinarum*, but it also allows the easy formation of a uniform thin film and thin film patterning through masking. Figure 6 shows a photograph of the dip coating apparatus. The dip coating apparatus has been placed inside a sound and vibration proof box to allow the fabrication of stable and uniform thin films. A glass substrate coated with transparent, conductive ITO (Indium tin oxide) was cleansed with a UV ozone cleaner, and used as the substrate for immobilizing the bR. A Tris-HCl buffer solution (10 mM) was added to the bR suspension to control the coagulation of the protein. Figure 7 shows an ITO substrate being withdrawn from the bR suspension to fabricate a bR thin film using the dip coating method. The thickness of the film can be controlled in this process by adjusting the speed at which the substrate is withdrawn

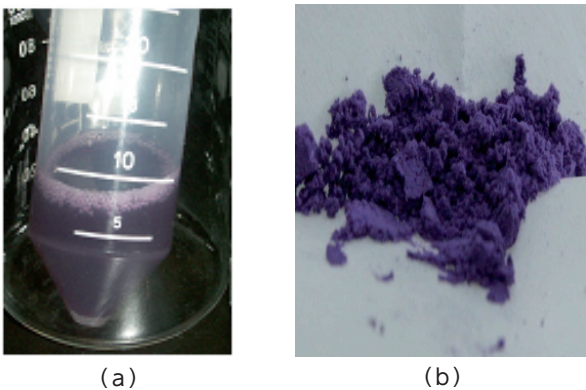


Fig. 4 (a) bR purple membrane suspension, and (b) freeze-dried powder, obtained from refining cultivated *Halobacterium salinarum*

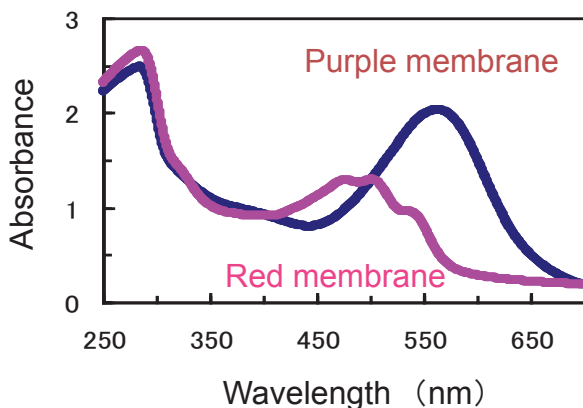


Fig. 5 Optical absorption characteristics of the bR purple membrane

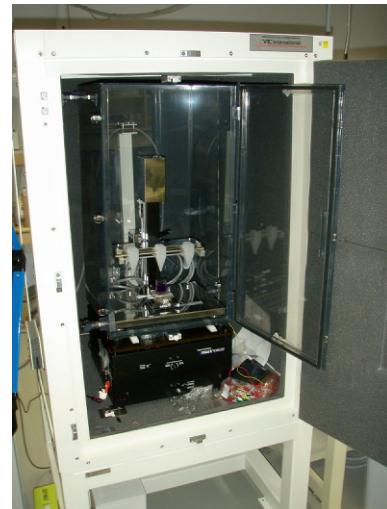


Fig. 6 Dip coating apparatus placed inside a sound and vibration proof box

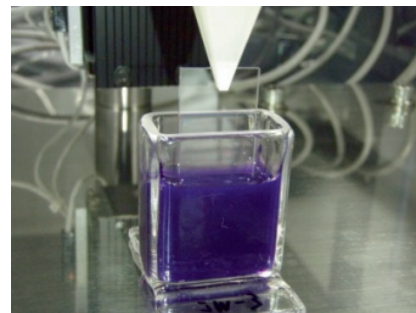


Fig. 7 Fabricating a bR thin film using the dip coating method (withdrawing an ITO substrate from the bR suspension (purple))

from the suspension, and we measured the thickness of the films at different speeds. The concentration of the bR suspension was set at 5 mg/ml, and the ITO substrate was withdrawn at different speeds between 0.01 and 10 mm/sec to create numerous bR thin films, the thicknesses of which were measured using an optical profiler (NewView™ 6200 of ZYGO). Figure 8 shows the relation between the film thickness and the speed at which the substrate is withdrawn from the suspension on a graph with logarithmic scales. The parameters in the dip coating method are the concentration and viscosity of the solution, and the speed at which the substrate is withdrawn^{[18][19]}. If the concentration and temperature of the bR suspension are constant, the film thickness increases with an increase in the speed at which the substrate is withdrawn from the suspension, in proportion to the square root of the speed. According to these experimental results, it is possible to control the thickness of the bR thin film within a range of 4–50 nm by controlling the speed at which the substrate is withdrawn from the suspension. Because the bR purple membrane is around 4 nm in thickness, it is possible to control the thickness of the thin film within a range of around 1–12 layers. The thickness of the thin film also depends on the concentration of the bR suspension, while it was possible to create the most uniform thin films with a concentration of 5 mg/ml. Next we used an AFM (Atomic Force Microscope) to examine the bR thin films we created (withdrawn at a speed of 0.1 mm/sec). Figure 9 is an AFM image of the bR purple membrane, and it shows circular or elliptical purple membranes with diameters of around 0.5 – 1 μm . It is thought that these purple membranes become layered over one another, forming the bR thin films made through the dip coating method.

4 Preparing a photoresponse cell, and its time response characteristics

We prepared a photoresponse cell using a bR thin film made through the dip coating method, and evaluated the characteristics of its time response to irradiated light. Figure 10(a) shows the structure of the photoresponse cell with a bR thin film made by dip coating, and (b) shows its external appearance. A bR thin film has been formed on one of two ITO substrates facing each other, and an electrolyte solution (KCl, 0.2 M, pH7.2) is enclosed using an O-ring (1.9 mm in thickness with an internal diameter of 9.8 mm). The acrylic plates on both sides are for fixing the opposing ITO substrates on. Figure 11 shows the

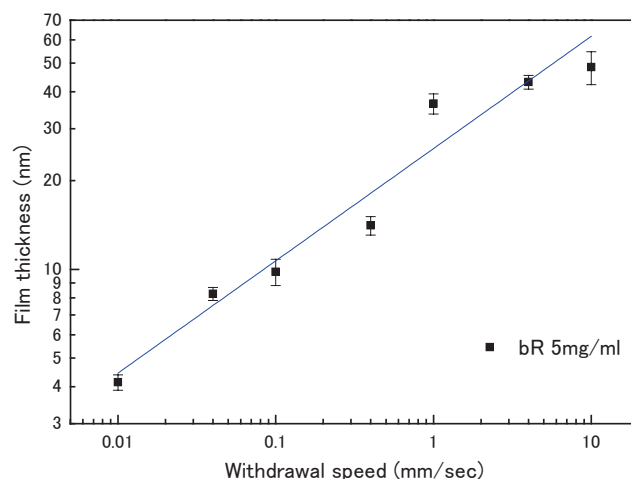


Fig. 8 Relationship between the film thickness and the speed at which the substrate is withdrawn from the suspension (bR concentration: 5 mg/ml)

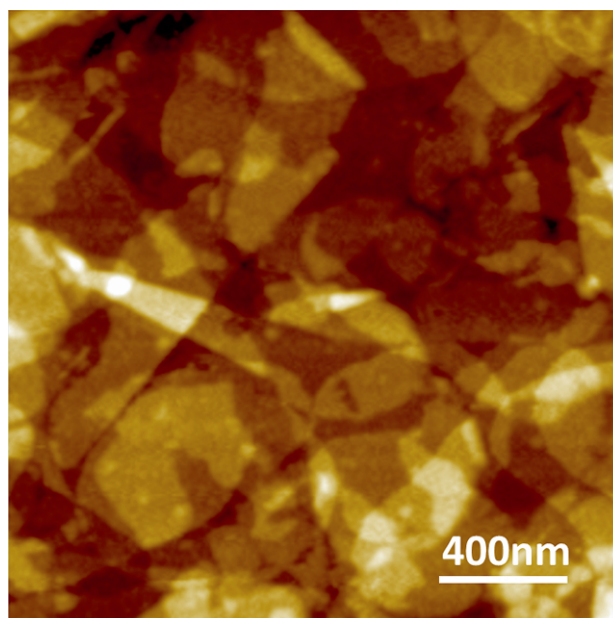


Fig. 9 AFM image of bR purple membranes

experimental setup for evaluating the photoresponse characteristics of the prepared bR cell. Because the absorption maximum of bR in its ground state is observed at a wavelength of around 570 nm, we used an optically pumped semiconductor laser (Sapphire 568 of Coherent, Inc.) as the light source, which emits yellow light at that wavelength. An AO modulator was used to control the ON/OFF of the laser light emission, and a beam expander was used to irradiate the entire light receiving area of the bR cell. The photocurrent output from the bR cell was measured with an oscilloscope using a current-to-voltage conversion amplifier (IV amplifier). The intensity of the irradiated light was monitored using a photodiode. Figure 12 shows the photocurrent obtained when a cell made of

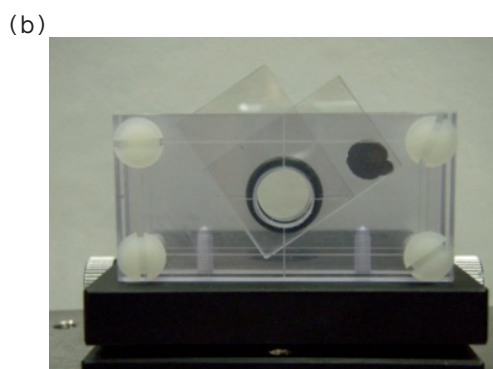
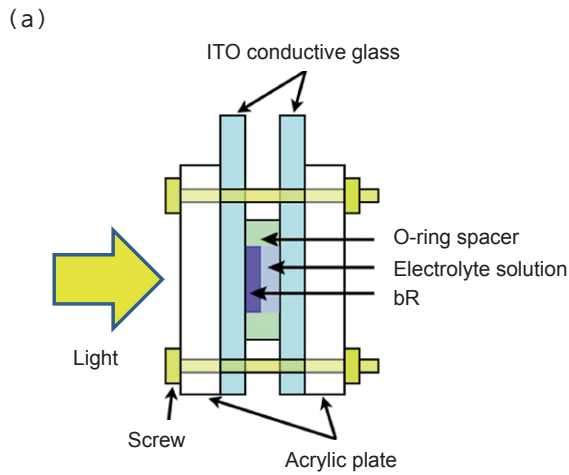


Fig. 10 (a) Structure of the photoresponse cell with a bR thin film, and (b) its external appearance

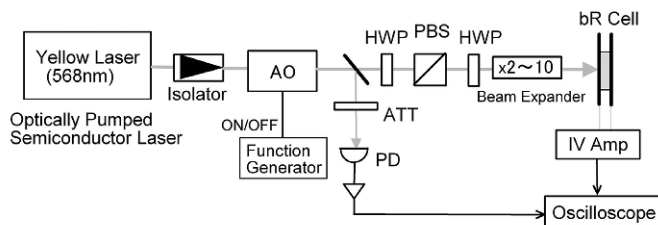


Fig. 11 Experimental setup for evaluating the photoresponse characteristics of the bR cell (AO: acousto-optic modulator, PD: photodiode, HWP: half-wave plate, PBS: polarizing beamsplitter, ATT: optical attenuator, IV Amp: current-to-voltage conversion amplifier).

bR thin film of 36 nm in thickness, fabricated by withdrawing the substrate at a speed of 1 mm/sec during dip coating, is irradiated with ON/OFF (70 mW) light of 1 Hz. It shows the time-differential response characteristics in accordance with the ON/OFF changes during irradiation, with a transient photocurrent of -180 nA while the light is on, and $+130$ nA when the light is off. This time-differential photocurrent is mainly due to the mechanism of the proton (H^+) pump function of the bR, and it is believed to be caused by changes in the pH on the electrode surface, which is dependent on the release and

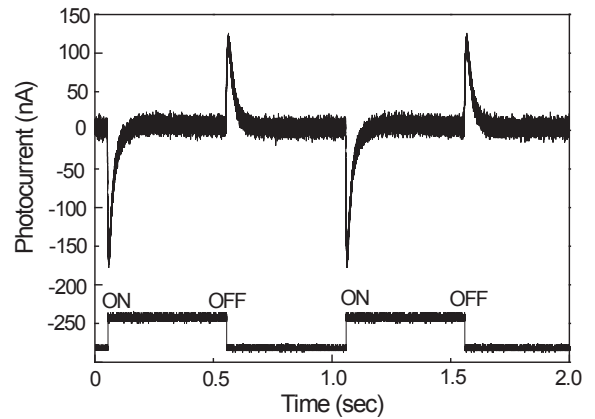


Fig. 12 Time-differential-type photocurrent response of the bR cell (the lower signal is the ON/OFF reference output from the photodiode)

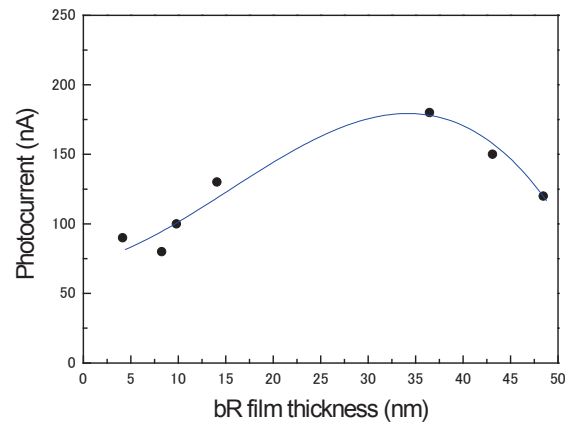


Fig. 13 Dependence of the photocurrent on the film thickness (when the light is turned on)

uptake of protons, just as in other cases where bR thin films made by other methods were used^{[20]-[22]}. The photocurrent response flowed in the direction of the cathode from the electrode on which the thin film was immobilized when the light was turned on, and in the direction of the anode when the light was turned off. Next we created bR thin films of varying thicknesses by changing the speed at which the substrate was withdrawn during dip coating, and evaluated the dependence of the photocurrent (when the light is turned on) on the film thickness. Figure 13 shows the dependence of the photocurrent, generated by the bR cell, on the film thickness. The photocurrent increases with an increase in the thickness of the film and reaches its maximum at a thickness of 36 nm, after which it begins to decline. This means that because the bR purple membrane has a thickness of around 4 nm, the current continues to increase until there are 9 layers of membranes. The mechanism behind this photocurrent is based on the directional proton pump function of the bR, and it is due

largely to the polar orientation of the bR. Because the photocurrent increases almost in direct proportion to the film thickness, it is believed that in accordance with the speed at which the substrate is withdrawn, the bR thin film is formed during dip coating in a way that the polar orientation becomes uniform to a certain extent^{[23][24]}. Furthermore, it is possible that the decline in current above a film thickness of 36 nm is due to irregularities in the polar orientation during formation of the thin film, and an increase in the equivalent resistance due to the thickness of the thin film, among other factors^[25].

5 Creating a visual function with bipolar cells

In the visual system of humans and animals optical signals entering the retina are converted to electrical signals by photoreceptor cells, which then pass through horizontal cells, bipolar cells, etc., to arrive at ganglion cells. As this happens, not all optical signal information is transmitted to the brain to be processed, but visual information processing takes place beforehand. The contour extraction (edge detection) function is an example of this preprocessing. The contour extraction function is an important part of information processing in image recognition of the visual function. When an area on the retina known as the visual receptive field is stimulated by light, the ganglion cells respond with excitation or inhibition. This emphasizes the contours of images before the information is transmitted to the brain. The photoresponse signal from the bR cell shows the same time-differential response waveform as that seen in the eyes of humans and animals, allowing the creation of a simulated visual receptive field by making clever use of the properties of excitation and inhibition^{[26][27]}.

In fabricating thin films through dip coating, patterning of the bR thin film is easily made by withdrawing an ITO substrate that has undergone masking treatment. In addition, when one focuses on the fact that the direction of the photoresponse current from the bR cell at the time of irradiation is rectified in the direction of the cathode, it becomes possible to build a bipolar photoresponse cell by fabricating bR thin films on both opposing ITO substrates. Furthermore, by implementing selective patterning on the bR thin films of both substrates, it becomes possible to create extremely simple, equivalent excitatory and inhibitory regions. Therefore, a bipolar bR cell like this can function as a biomimetic sensor that

simulates the function of the visual receptive field, which forms the fundamental unit of visual functions. Figure 14 shows a diagram of a bipolar photoresponse cell created using bR thin films. As shown in Fig. 14(a), a bR thin film has been formed in the central region (excitatory region) on the substrate on the upper side of the cell, and in regions on both sides (inhibitory regions) of the substrate on the lower side of the cell. In our experiments, our objective was to evaluate the fundamental characteristics of the sensor we created, and we examined its edge detection characteristics by moving a shading plate horizontally (from left to right in the diagram), while irradiating it with a laser beam (with a wavelength of 568 nm) expanded with a beam expander. Figure 14 (b) is the top view of the structure of the cell, with the central region being the excitatory region (15 × 6 mm²), and the regions on both sides being the inhibitory regions (2 × 15 × 3 mm²). Because the photocurrent generated by each of the regions during irradiation flows in the direction of the cathode, the output current between the cell electrodes is calculated using the current from the excitatory region (positive) and the current from the inhibitory regions (negative). As a result of the edge detection when the shading plate is moved, a photocurrent (central part) is obtained that is relative to the position of the edge, as shown in Fig. 14(c). Figure 15 shows the characteristics of edge detection in the

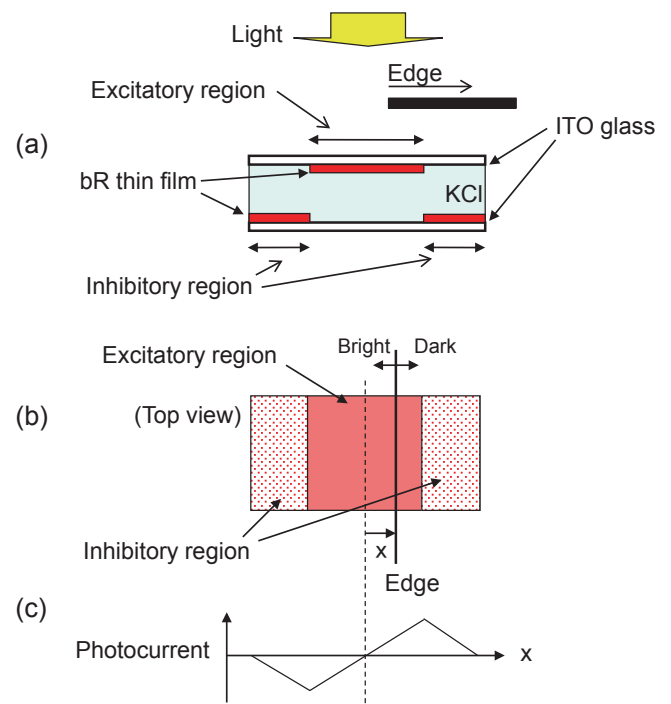


Fig. 14 Implementation of a visual receptive field using a bipolar bR cell: (a) structure of the cell (cross section), (b) structure of the cell (top view), (c) Conceptual diagram of edge detection.

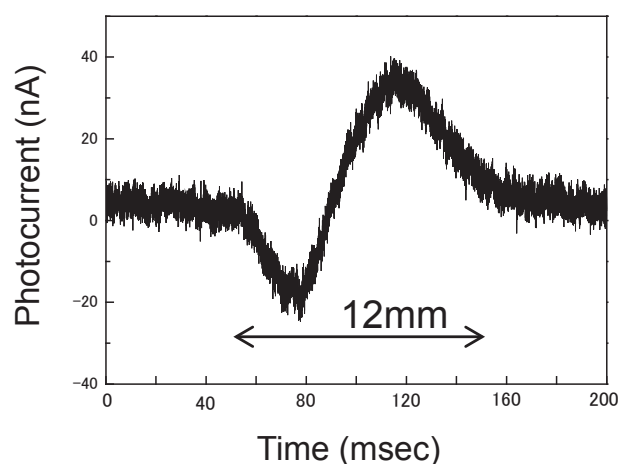


Fig. 15 Characteristics of edge detection in the bipolar bR photosensor (speed at which the edge moves: 120 mm/sec)

bipolar bR photosensor we created, when the edge moves at a speed of 120 mm/sec. The moving edge is detected and a photocurrent is generated in accordance with its displacement. We interpret this to mean that the visual receptive field function was simulated along a single dimension.

6 Conclusions

In this paper we have discussed the fabrication of thin films of bacteriorhodopsin, a photoreceptor membrane protein, through the dip coating method, and its application in biomimetic sensing. The apparatus for dip coating can be based on existing fabrication technology, and it allows for straightforward control of film thickness. Operation at room temperature makes it especially suitable for biomaterials. The maximum photocurrent obtained under the thin film fabrication conditions of our experiment was 180 nA, but it was confirmed that the photocurrent may increase to around 800 nA depending on the concentration of the bR suspension. This may be due to coverage by the purple membrane within the thin film; there is a need for further optimization of the conditions under which films are formed. The photocurrent may also be boosted by stacking of the bR cell, which can be used as a trigger for starting up systems, making it possible to build a photoresponse sensor that does not require a power source. Unlike conventional sensors, the photoresponse output of bR is a time-differential response, the same as that of humans and animals. It does not respond to constant light, responding only when there is a change, making it ideal for detecting the movement of objects. We demonstrated that it is possible to create an

artificial visual receptive field function by combining a simple bipolar-type structure with a patterned bR thin film. With this structure, it is possible to detect the direction of movement of objects by giving anisotropy to the patterning form and arrangement, and we are currently considering the possibility of its application in robot vision^[28]. It is also possible to create bR variants with different photoresponse characteristics through genetic recombination technology^[29], and we are developing photosensors that detect optical flow (the velocity field of objects that move relative to one another) through a combination of these variants^{[30][31]}. The visual information system found in insects has been optimized in the course of evolution to instantaneously perceive information on the movement of objects around the insect, or its own movement, and it uses optical flow as visual information. On the other hand, in an autonomously moving robot operating in the real environment, it is important to create a specialized system for controlling movement to avoid dangers, and it is also necessary to design a visual system that overcomes the limitations of power consumption, size, cost, etc. There is expected to be an increase in demand in the future for autonomous moving robots that are remotely controlled through networks, and we believe that the development of insect mimetic sensing technology, which simulates optical flow detection in insect visual systems, will lead to a breakthrough in overcoming the limitations mentioned above.

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