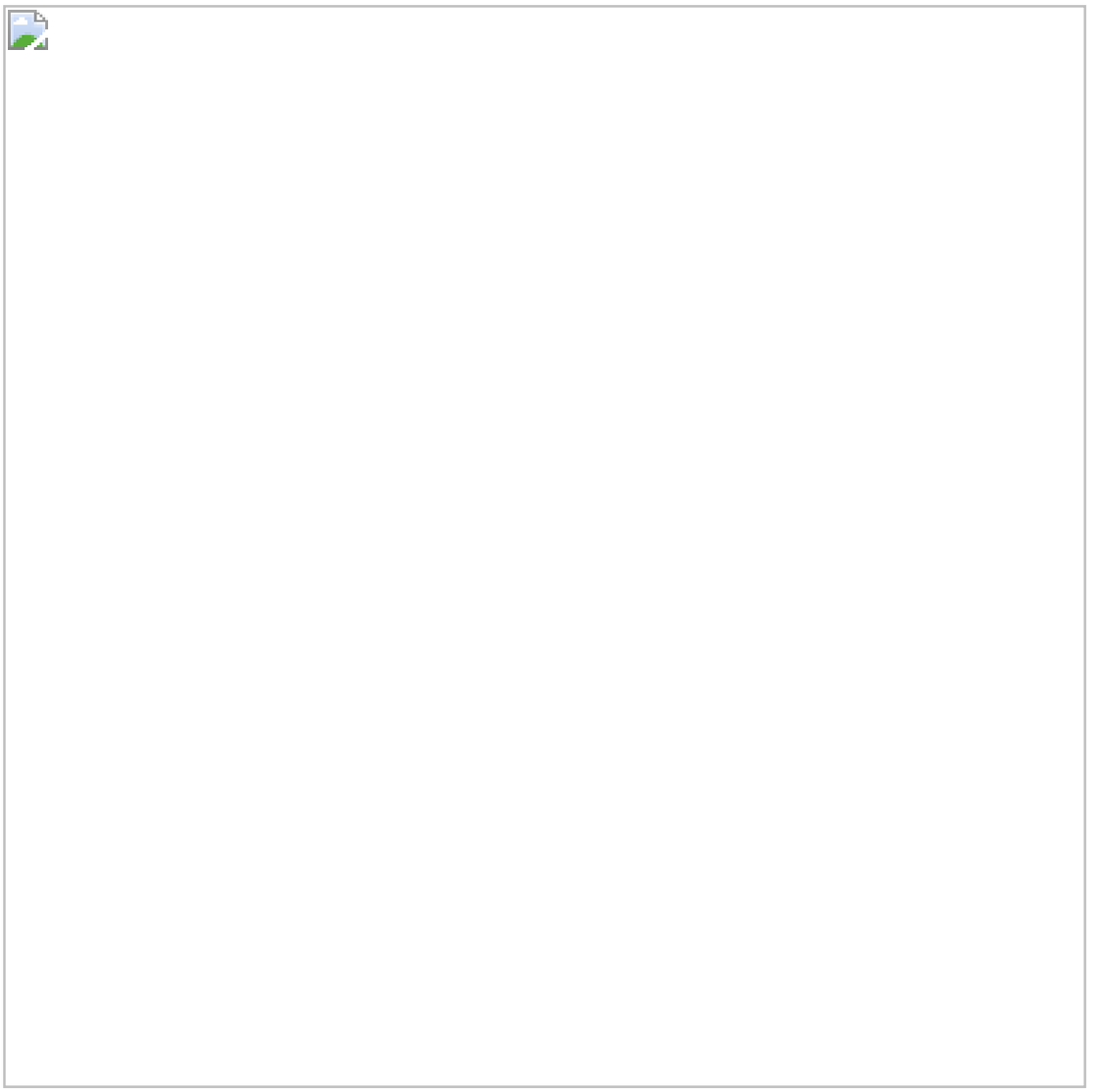

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Visualization of Retinal Proteins in the Cerebral Ganglion of Ascidian, *Halocynthia roretzi*

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ABSTRACT—Retinal proteins in the cerebral ganglion of the ascidian, *Halocynthia roretzi*, were successfully visualized and their localization was determined by the time-resolved fluorescence difference imaging method. This visualizes retinal proteins in the tissue even though the concentration of the retinal proteins is low and there is considerable endogenous fluorescence. Heterogeneous retinal proteins in the same tissues could be distinguished by differential sensitivities to photobleaching, standardized by rhodopsin and retinochrome in octopus retina. Retinal proteins in the ocellus of ascidian larva, which is composed of only about 20 photoreceptor cells, were successfully visualized.

Retinal proteins in the cerebral ganglion of adult *Halocynthia roretzi*, localized mainly at the surface of the anterodorsal root and the posterodorsal root. In the cross sections along the anteroposterio axis of the cerebral ganglion, the cells bearing retinal proteins were found in the peripheral cellular cortex mainly at the dorsal surface. Close localization of retinal protein and GnRH bearing cells suggests that retinal protein may trigger the biological clock for spawning in this ascidian.

INTRODUCTION

Successful reproduction in cross-fertilizing organisms depends on synchrony among members of the population. As reproductive cycles are correlated with temperature, day length, lunar periods and tide level, physical parameters such as temperature, light, gravity and pressure are assumed to be causative factors that entrain the cycles (Svane and Young, 1989)

Invertebrate embryologists have long known that a number of ascidians spawn at an fixed latency period after sunrise (Svane and Young, 1989; Lambert and Brandt, 1967). The timing of gamete or larval release is controlled by light in all species that have been experimentally investigated. In general, spawning occurs in response to light following an appropriate dark adaptation. The duration of light exposure required, which has been called the latency period (Lambert *et al*., 1981), varies among species. Numakunai and Hoshino (1980) found that there are three types of *Halocynthia roretzi* in Mutsu Bay which show different spawning seasons and spawning times; morning type, spawning 4 hr after sunrise (November); noon type, spawning 6 hr after sunrise (January); and evening type, spawning 10 hr after sunrise (December). These phenomena can be reproduced in the laboratory. For example the noon type spawns 6 hr after exposure to light following 6 hr of darkness at 12°C in the laboratory, whenever starts. These results suggest that the biological clock for spawning of ascidians is initiated by light and latency time is specific to the type of ascidians.

Some physical parameters controlling the release of gametes in several ascidians were studied, temperature, minimum dark adaptation period, the time required for spawning (West and Lambert, 1976), and the action spectrum for spawning (Lambert and Brandt, 1967). Though the photoreceptor responsible for spawning was proposed to be cytochrome C by the action spectrum for spawning of *Ciona* (Lambert and Brandt, 1967), there has been no direct evidence for the involvement of a hemoprotein in photo-induced spawning behavior.

The conversion of light into neural signals has been the best studied for the photoreceptor cells of the retina. However, animals show various nonvisual photoreceptive tasks. Recently extraretinal photoreceptors were found to be exist in the pineal (Okano *et al*., 1994), deep brain (Wada *et al*., 1998; Yoshikawa *et al*., 1998), and the dermal melanophores (Provencio *et al*., 1998) in skins. Most extraretinal photoreceptors contain retinal proteins which suggests their involvement in controlling for circadian rhythms, seasonal behavior for sexual reproduction, photic control of skin pigmentation, and papillary aperture.

Previous work shows retinal isomers extracted from the cerebral ganglion of *Halocynthia roretzi* undergo interconversion between all-*trans* form and *11-cis* form by illumination of blue and orange light, respectively. From these results, Kajiwara *et al*. (1990) proposed that the cerebral gan-

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glion is the photoreceptor that controls gamete release in the adult ascidian.

We have developed a highly sensitive method to visualize retinal proteins by the time-resolved difference fluorescence imaging method. The aldimine of a retinal Schiff base is reduced by sodium borohydride converting the retinal protein to N-retinyl opsin which is relatively stable and emits visible fluorescence at 450 nm upon irradiation with 360 nm light (Morton and Pitt, 1955; Bownds and Wald, 1965). This reaction was successfully applied to visualize the retinal proteins in the retinas of several animals (Eakin and Brandenburger, 1978; Ozaki *et al*., 1983). However, this method has the following disadvantages, (1) Fluorescence of the N-retinyl protein is easily bleached by actinic light. (2) It is hard to distinguish the fluorescence of the N-retinyl protein when the endogenous fluorescence of the organs is high. Thus it is hard to visualize retinal proteins in extraocular photoreceptors in which the retinal is low and there is considerable endogenous fluorescence.

Since the bleaching rate of N-retinyl protein by near UV actinic light is much higher than that of the endogenous fluorescence, the time-resolved difference fluorescence imaging method enabled us to visualize the retinal protein, while endogenous fluorescence was eliminated. First, we standardized this method by visualizing retinal proteins in octopus retinas which had been studied by conventional methods (Ozaki *et al*., 1983). We next applied this method to visualize retinal proteins in the ocellus of ascidian larvae which are composed of about 20 retinal cells (Nicol and Meinertzagen, 1991). This method allowed us to successfully visualize and determine the localization of retinal protein in the cerebral ganglion of the adult ascidian. This ganglion is expected to contain the photoreceptor for the biological clock controlling sexual reproduction.

MATERIALS AND METHODS

Animals

Living *Octopus vulgaris*, was purchased from a local fishery (Tokusui Shoji, Akashi, Japan). Retinas were isolated from the octopus eyeballs.

Ascidian, *Halocynthia roretzi* was purchased from fishermen in Sameura Bay at Miyagi, where the animals were maintained in running artificial seawater (Marine Art BR, Senjyu, Osaka, Japan) at 4–8°C. Samples used for analyses were the cerebral ganglia, located between the base of the oral and the atrial siphons.

Eggs and sperm were obtained surgically from the gonad. Eggs were fertilized by sperm from another animal in a petri dish. The fertilized eggs were cultured at 12°C in artificial seawater until tadpole larvae hatched.

Preparations

Organs were transferred into a Bright Cribbed embedding compound (Bright Instrument Co. Ltd., England), and immediately frozen at liquid nitrogen temperature. All the following operations were carried out under dim red light. The frozen samples were sectioned at 20 μm with a Bright model 5030 rotary microtome set up in a Bright model 5030 cryostat adjusted to –16°C. Each section was mounted on a poly-lysine coated glass slide and air dried for 4 hr at about 20°C.

In order to visualize intrinsic retinal protein in the specimen, the following four different preparations were used for all tissues studied (octopus retina, ascidian larva, cerebral ganglia of adult ascidian). 1) Non-treated specimen. 2) The specimen was immersed in a 0.2% sodium borohydride for 10 sec at 4°C, rinsed gently with water. Labile retinal protein such as retinochrome was reduced. 3) The specimen was treated with a 20% aqueous solution of formaldehyde for 3 min at room temperature and washed with water. 4) After treating the specimen with formaldehyde, the denatured retinal protein was then reduced by 0.2% sodium borohydride for 10 sec at 4°C.

Measurements

Since the retinal proteins are abundant in the retina, the fluorescence of the N-retinyl protein in the retina was recorded by a conventional photographic camera (Ozaki *et al*., 1983). However, this measurement can not be used to visualize extraretinal photoreceptors in which retinal protein levels are low and endogenous fluorescence is high. Moreover, the fluorescence from the N-retinyl proteins is easily bleached by actinic light. In order to visualize only retinal proteins while eliminating endogenous fluorescence from such tissues, we developed a time-resolved difference fluorescence imaging method. Fluorescence images of the specimen were observed using Olympus IX-70 microscope equipped with an intensified CCD (ICCD) camera. To excite the N-retinyl protein in the specimen, near UV (330 nm–385 nm) actinic light from a 75 W high-pressure xenon lamp (Ushio inc.) was isolated by combining a BP330-385 filter and a DM-400 dichroic mirror. The fluorescence images of the tissues were observed through a BA420 filter to exclude reflected light upon excitation. After excitation of the N-retinyl protein by near UV actinic light was started, then images of the tissues were taken every 30 sec with an exposure of 1 sec. When the fluorescence image right after measurement (1 sec exposure) was subtracted from that at a given time (intensity of the fluorescence of the N-retinyl protein decay to ca. 80%) by ARGUE 50 (Hamamatsu Photonics), a time-resolved difference fluorescence image was observed.

RESULTS

Retinal proteins in octopus retina

Histochemical localization of the two distinct retinal proteins, rhodopsin and retinochrome, in the octopus retina has been studied by fluorescence microscopy with conventional photography (Ozaki *et al*., 1983). Before visualizing the novel retinal protein in the cerebral ganglion of the ascidian, retinal protein image of octopus retina was studied using the time-resolved difference fluorescence imaging method. As shown in Fig. 1A, the octopus retina is composed mainly of a large number of visual cells, each of which is divided into an inner and outer segment. The inner segment contains many lamellated bundles of retinochrome-bearing membranes, while the outer segments contain rhabdomeres of rhodopsin-bearing membranes and the black-pigment layer. Fig. 1B shows the fluorescence image of non-treated cryosection excited with a near UV actinic light observed with the ICCD camera with a 1 sec exposure. Weak fluorescence was observed over the entire area. When the specimen was treated with sodium borohydride, a distinct band with strong fluorescence was observed in the inner segment of the retina as shown in Fig. 1C, which is assigned to retinochrome in the inner segment of octopus retina. Fig. 1D shows the fluorescence image of a cryosection which was treated by formaldehyde before being reduced with sodium borohydride. The apparent fluorescence was reduced

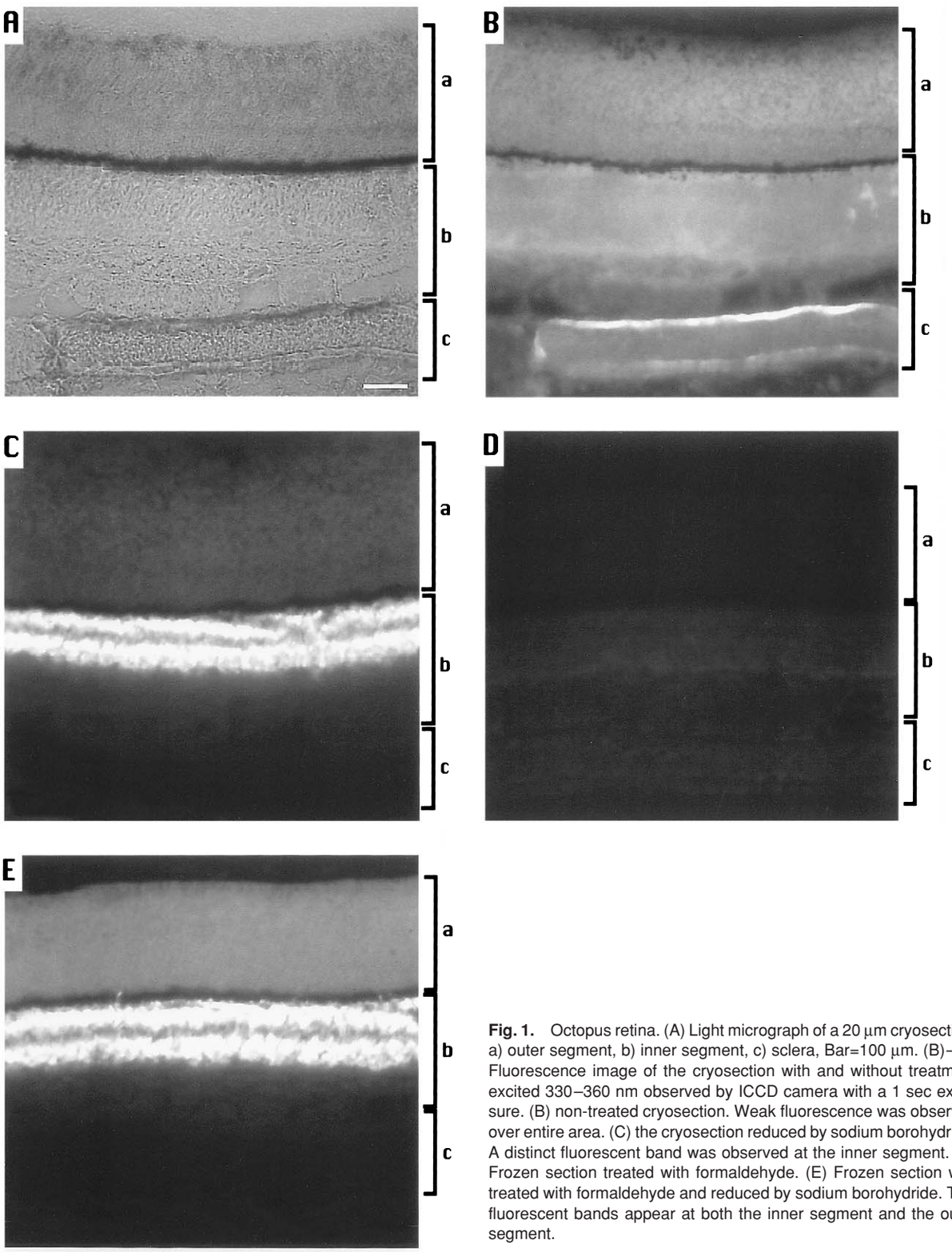

Fig. 1. Octopus retina. (A) Light micrograph of a 20 μm cryosection. a) outer segment, b) inner segment, c) sclera, Bar=100 μm. (B)–(E) Fluorescence image of the cryosection with and without treatment excited 330–360 nm observed by ICCD camera with a 1 sec exposure. (B) non-treated cryosection. Weak fluorescence was observed over entire area. (C) the cryosection reduced by sodium borohydride. A distinct fluorescent band was observed at the inner segment. (D) Frozen section treated with formaldehyde. (E) Frozen section was treated with formaldehyde and reduced by sodium borohydride. Two fluorescent bands appear at both the inner segment and the outer segment.

compared to Fig. 1B, because soluble fluorescent materials were washed out by the treatment and additional fluorescent species did not appear with denaturation. Fig. 1E shows fluorescence image upon treatment of octopus retina with formaldehyde and sodium borohydride. Two fluorescent bands were seen in the rhabdomal layer, a thin band appeared at the

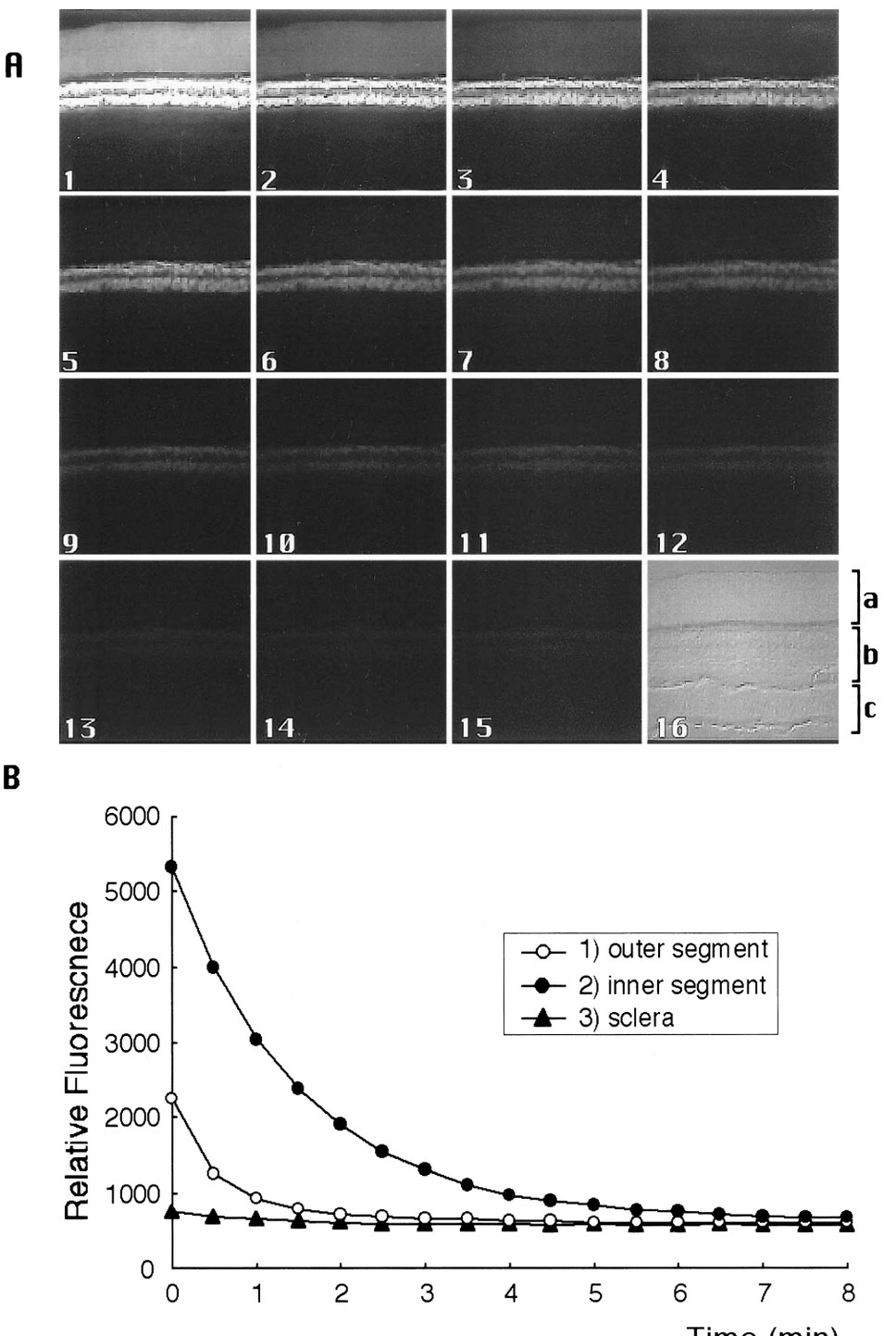

Fig. 2. (A) Time sequence of the fluorescence images of octopus retina treated with formaldehyde and borohydride under actinic light as observed by ICCD camera. Each image was exposed to ICCD camera for 1 sec. 1) right after exposure ~ 15) after 7 min illumination. The time interval between each panel was 30 sec. 16) a light micrograph. a) outer segment, b) outer segment, c) sclera. (B) Time courses of intensity of the fluorescence at the spots of the inner segment (1), the inner segment (2) and the sclera (3), respectively. The life time of decay in intensity of the fluorescence at different layers was different. Though the fluorescence from the outer segment (rhodopsin) fade within 3 min, it took more than 6 min for the fluorescence to fade from inner segment (retinochrome). Fluorescence of the sclera did not change during exposure to light.

inner segment due to reduced retinochrome, whereas a thick band appears in the outer segment due to reduced rhodopsin as previously assigned by Ozaki *et al*. (1983). They described that observed fluorescence of the N-retinyl protein from these retinal proteins faded rapidly under UV light, and disappeared after 1 min.

We measured the fluorescence bleaching process of the N-retinyl protein in the reduced octopus retina. Fig. 2A showed the time sequence of fluorescence images of reduced octopus retina under actinic light. Each image was exposed to the ICCD camera for 1 sec and the time interval between each panel was 30 sec. The first panel is the same as Fig. 1E, that is, two fluorescent bands appear in the retina, a thin band due to retinochrome and a thick band due to rhodopsin. During the course of the measurements, the intensity of the fluorescence from both bands decreased. The fluorescence from the outer segment (rhodopsin) faded much faster than that from the inner segment (retinochrome).

Fig. 2B shows the time courses of the fluorescence intensity at the spots of the outer segment, the inner segment, and the sclera. Though the fluorescence from the outer segment (rhodopsin) faded within 3 min, it took more than 6 min for the fluorescence from the inner segment (retinochrome) to fade. Fluorescence of the sclera did not change with time. These results suggest that the fluorescence of reduced retinal proteins was more photo-labile than the endogenous fluorescence from the tissues under the present conditions. Moreover, rates of bleaching of fluorescence of the N-retinyl protein from rhodopsin ($k=0.016\ sec^{-1}$) and retinochrome ($k=0.010\ sec^{-1}$) were obtained in the same tissue gave different values. This different photosensitivity for the N-retinyl protein from different retinal proteins enabled us to distinguish each with the time-resolved difference fluorescent imaging.

Fig. 3A shows the retinal protein image of octopus retina which was constructed by subtracting panel 15 (after 7 min illumination) from panel 1 (right after illumination). Both rhodopsin in the outer segment and retinochrome in the inner segment were well visualized. Since the fluorescence from the outer segment (rhodopsin) faded within 3 min, an image constructed by subtracting panel 15 (7 min illumination) from panel 5 (2 min illumination) gave selectively exhibited retinochrome in the inner segment.

Larval ocellus retinal proteins

The prosencephalon of the larva contains a single ventricle, along the inner walls of which lie two sensory organs; the otolith and the ocellus. The otolith is assumed to be involved in geotactic responses (Svane and Young, 1989). The ocellus is a photoreceptor organ whose morphology has been well studied, but there is no direct evidence for retinal proteins.

Visualization of retinal proteins in the ocellus was studied by the time-resolved difference fluorescence imaging method for the different preparations of cryosection of larva. Though fluorescence of specimen was observed for all preparations treated with/without formaldehyde and/or sodium borohydride described in Materials and Methods, time-resolved difference fluorescence image was only observed for formaldehyde and sodium borohydride treated specimen. Fig. 4A shows the fluorescence image of the formaldehyde and sodium borohydride treated cryosection of the larva right after exposure to actinic light. Fig. 4B shows the fluorescence image of the same specimen after 5 min exposure to actinic light. Fig. 4C is the retinal protein image obtained by the difference between Fig. 4A and Fig. 4B. The fluorescence of the N-retinyl protein bleached rapidly and disappeared after 5 min.

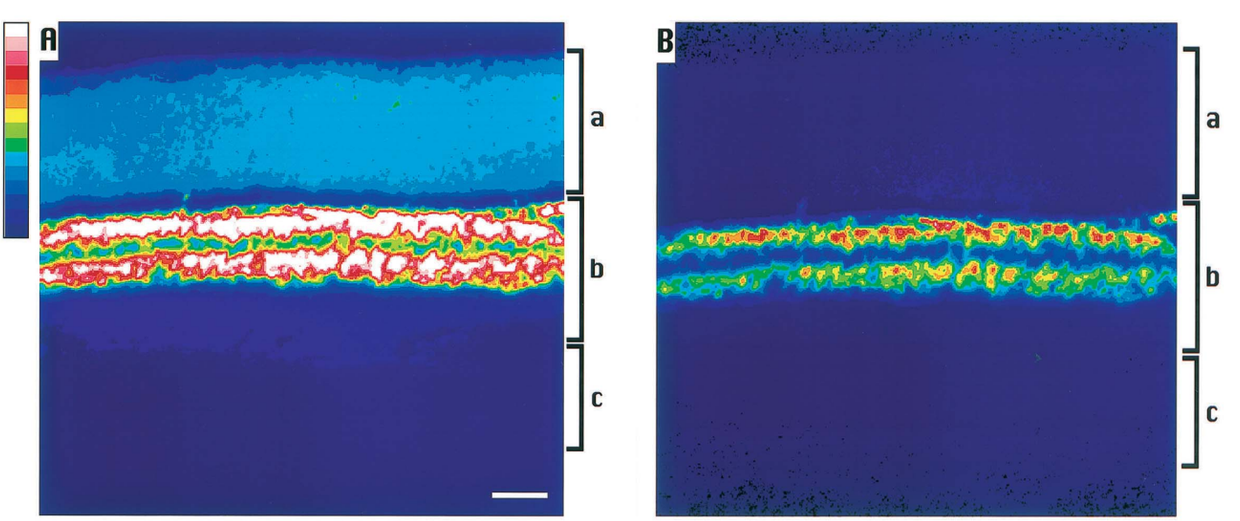

Fig. 3. The time-resolved difference fluorescent images of an octopus retina treated with formaldehyde and sodium borohydride. (A) Difference of fluorescence image between panel 1 (right after illumination) and panel 15 (after 7 min illumination) in Fig. 2A. Both rhodopsin in the outer segment and retinochrome in the inner segment were visualized. (B) Difference of the fluorescence image between panel 5 (2 min illumination) and panel 15 (7 min illumination). Since most of fluorescence from the outer segment (rhodopsin) has faded at 2 min, fluorescence from the inner segment (retinochrome) was selectively visualized.

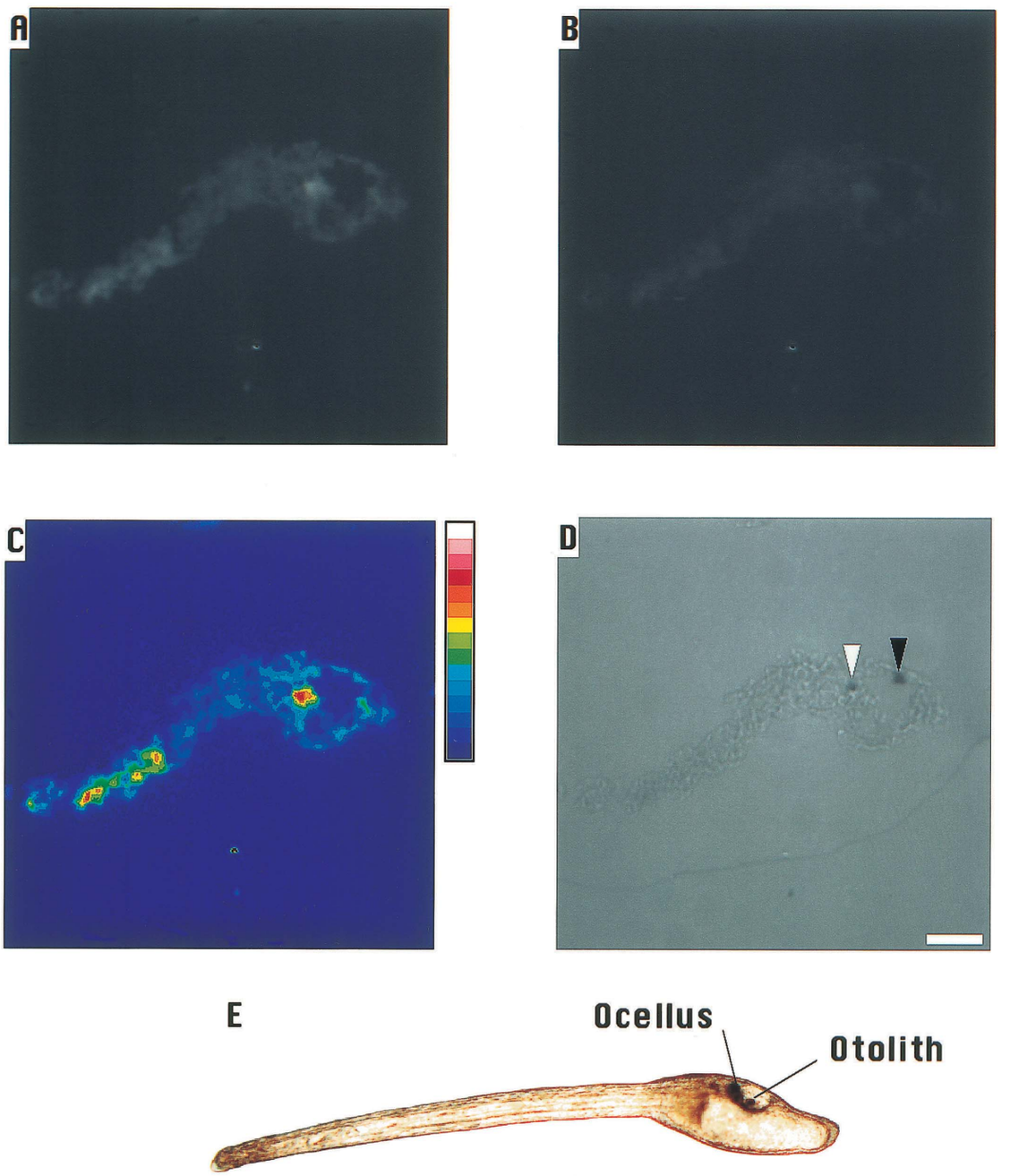

Fig. 4. Visualization of retinal protein in ascidian larva treated with formaldehyde and sodium borohydride. (A) The fluorescence image of the section of larva right after exposure to actinic light. (B) The fluorescence image of the same specimen after a 10 min exposure to actinic light. (C) The difference of the fluorescence images between A and B. Reddish color exhibit the localization of retinal proteins in the ocellus of the larva. (D) Light micrograph of the same cryosection. Black arrow head shows pigment of otolith, and white arrow head shows pigment of ocellus. Bar = 100 μm.

However, the fluorescence from the endogenous pigment decreases much slower than that of the N-retinyl protein. Thus, Fig. 4C shows the localization of the retinal proteins. Fig. 4D shows a light microscope image of the same cryosection. There are two pigment organs in the prosencephalon of the larva. Comparison of Fig. 4C and Fig. 4D shows that fluorescence of the N-retinyl protein is localized at posterior wall in the ventricle of the prosencephalon, that is the ocellus. Fluorescence was also found at the tail part of the larva, which is most likely retinal proteins. Identification of this retinal protein is open for further investigation.

Adult cerebral ganglion retinal proteins

The cerebral ganglion of *Halocynthia roretzi* is 10 mm long and about 0.5 mm in diameter. It is located in the body wall under the tunic between the base of the oral and atrial siphons. In order to visualize intrinsic retinal proteins in the specimen, the isolated ganglions were treated with/without formaldehyde and/or sodium borohydride described in Materials and Methods. The fluorescence image of the preparation was measured with the ICCD camera. Though fluorescence of specimen was observed for all preparations treated with/without formaldehyde and/or sodium borohydride described in Materials and Methods, time-resolved difference fluorescence image was only observed for formaldehyde and sodium borohydride treated specimen.

Fig. 5A shows the fluorescence image of the ventral side of the posterior half of the whole-mount preparation of the cerebral ganglion treated by sodium borohydride and formaldehyde right after exposure to actinic light (1 sec exposure to ICCD camera). The cerebral ganglion showed fluorescence over the entire area. Fig. 5B shows fluorescence images of the same preparation after 5 min exposure to actinic light. Fluorescence mainly decreased in the middle part. Fig. 5C is the time-resolved difference fluorescence image between Fig. 5A and Fig. 5B. Though the reduced ganglion shows that the fluorescence over the entire area (Fig. 5A), the time-resolved difference fluorescence image (Fig. 5C) shows that the fluo-

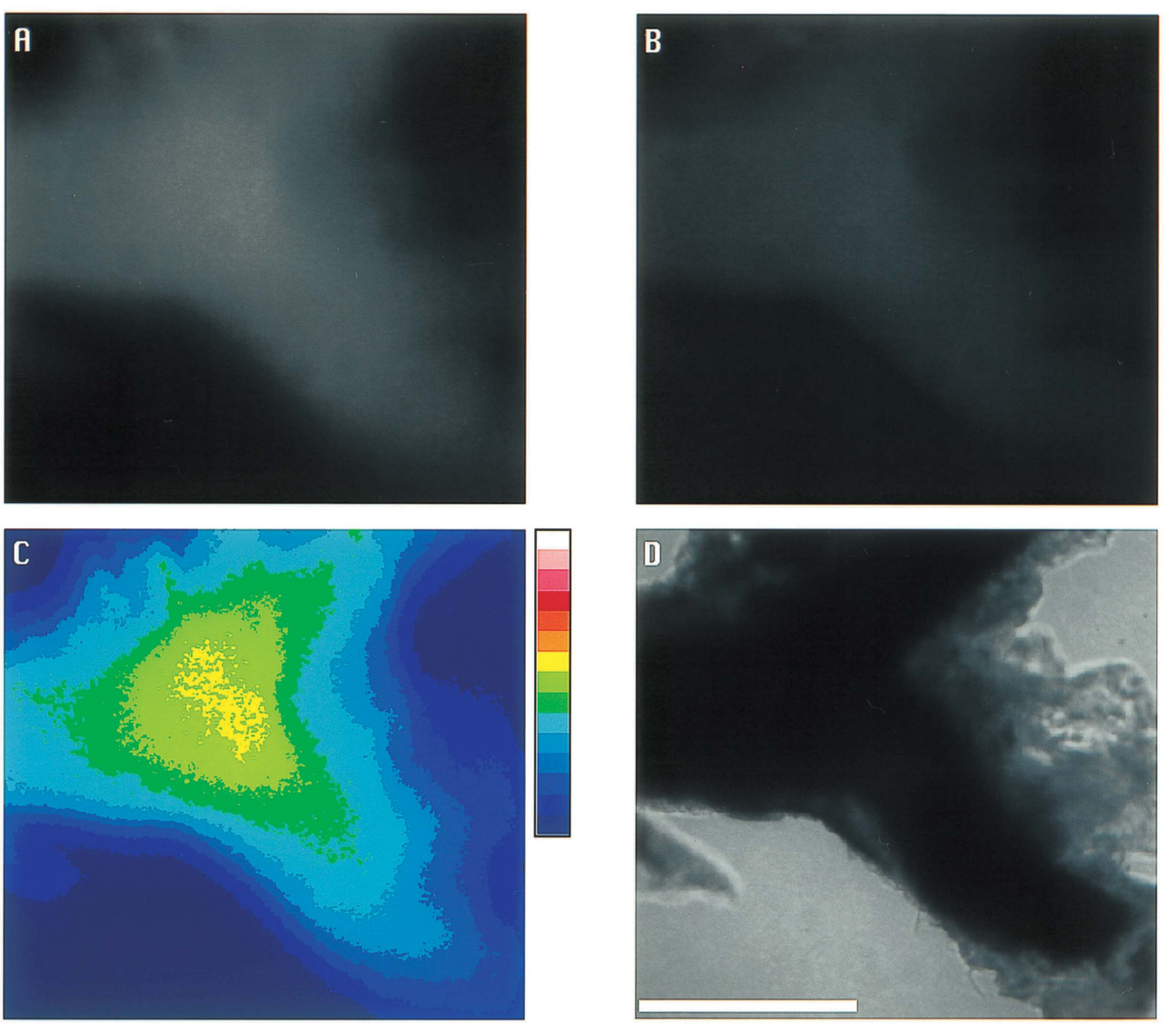

Fig. 5. Visualization of retinal proteins in the whole mount of the cerebral ganglion of the adult ascidian. (A) Fluorescence image of the ventral side of the posterior half of the whole-mount preparation of the cerebral ganglion right after exposure to actinic light (1 sec exposure to ICCD camera). (B) Fluorescence of the same preparation after 10 min exposure to actinic light. (C) The difference fluorescence image between A and B. (D) Light micrograph of whole mount preparation. Bar=1 μm.

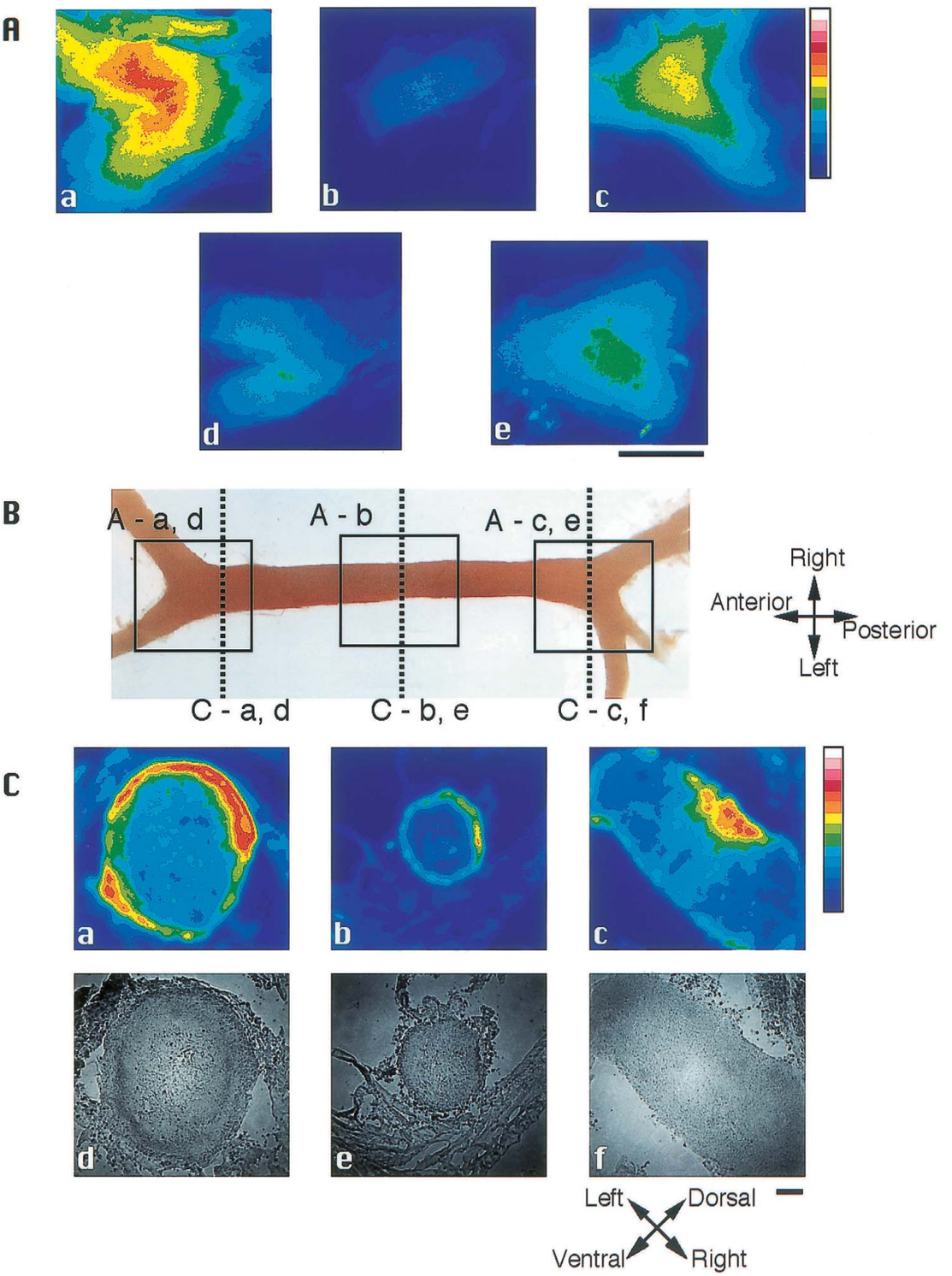

Fig. 6. Localization of retinal protein in the cerebral ganglion. (A) The time difference fluorescence images, a; anterodorsal surface, b; middle part of dorsal surface, c; the posterodorsal surface, d; the anteroventral surface, e; the posteroventral surface. Retinal protein was localized in two separated parts, the anterodorsal surface of the root between the right anterior nerve and left anterior nerve and the posterodorsal surface of the root between the right posterior nerve and posterior nerve. (B) Light micrograph of the whole mount preparation of the cerebral ganglion of *Halocynthia roretzi* viewed from the dorsal side. Square shows the areas of the image in Fig 6B. Dashed lines means the places of cryosection of Fig 6C. (C) Retinal protein images of cryosections of cerebral ganglion. a–c) Time difference fluorescence images of cryosection of the cerebral ganglion. d–f) Light micrograph of images corresponding to a to c. a) Fluorescence image of cryosection at the ventral side branch. b) Fluorescence image of cryosection at the middle part of the ganglion. c) Fluorescence image of cryosection at the dorsal side branch. Bar=100 μm.

rescence is located on the posterodorsal surface of the ganglion, surrounding the root between the right and left posterior nerves. Though the fluorescence images of the cerebral ganglion denatured by formaldehyde showed weak fluorescence over the entire area, there are no differences in time-resolved difference fluorescence imaging. These results strongly suggest that the fluorescence originated from the N-retinyl protein which was produced by reduction of retinal protein in the cerebral ganglion.

Localization of retinal proteins in the whole cerebral ganglion was investigated. Since the cerebral ganglion of *Halocynthia roretzi*, about 10 mm long and about 0.5 mm in diameter, is too large to be entirely imaged with the microscope, the localization of retinal protein was investigated for different parts of the ganglion. Fig. 6B shows a light micrograph of the whole mount preparation of the cerebral ganglion viewed from the dorsal side. Fig. 6A are the retinal protein images, B-a; anterodorsal surface, b; middle part of the dorsal surface, c; the posterodorsal surface, d; the anteroventral surface, e; the posteroventral surface. These results suggest that the retinal protein is localized at the surface of the anterodorsal root and the surface of the posterodorsal root.

In order to determine the localization of the retinal protein in a cross section of the cerebral ganglion, cryosections along the anteroposterio axis of the cerebral ganglion were reduced by sodium borohydride after denaturation by formaldehyde and time-resolved difference fluorescence imaging were observed as shown in Fig. 6C. The retinal proteins were found at the sheath of the cerebral ganglion, mainly in the dorsal side but less in the ventral side.

DISCUSSION

The time-resolved fluorescence difference imaging method was successfully applied to visualize retinal proteins in several tissues. This method was standardized with the octopus retina where the histochemical localization of retinal proteins has been well studied by conventional fluorescence microscopy (Ozaki *et al*., 1983). There are two different retinal proteins, rhodopsin in the outer segment and retinochrome in the inner segment. As shown in Fig. 3, the differences in photosensitivity for the bleaching of fluorescence enabled us to identify the localization of different retinal proteins in the same tissues.

Next we tested how a small number of cells can be visualized with this method. The ocellus of the ascidian larvae is composed of about 20 sensory cells whose morphology was well studied (Eakin and Kuda, 1971; Barnes, 1971; Kajiwara and Yoshida, 1985). Though the outer segment of the photosensory cells has typical photosensory morphology, with cilia with many lamella, there is no direct evidence for retinal protein. As shown in Fig. 4, we successfully visualized retinal proteins in the cells in the ocellus. This method proves to be highly sensitive. The existence of retinal proteins in the ocellus was also supported by our recent work on the photic behavior of the larvae of *Ciona intestinalis*, that is, the action spectrum for photophobic response corresponds to absorption spectrum of a vertebrate rhodopsin (Nakagawa *et al*., 1999).

The time-resolved fluorescence difference imaging method was used to visualize retinal proteins in the ascidian cerebral ganglion. Retinal proteins in the cerebral ganglion were localized mainly at the surface of anterodorsal root and the posterodorsal root. Fewer retinal proteins were found at the surface of anteroventral root and at the posteroventral root of the ganglion. However, there are few retinal proteins found in the middle part. These results were further confirmed by visualization of the retinal protein in the cross section of the cerebral ganglion along the anteroposterio axis. The ascidian cerebral ganglion consists of a central neuropile and peripheral cellular cortex. Fig. 6C shows that retinal proteins in the cross section of the ganglion were found to localize outside of the peripheral cellular cortex, mainly at dorsal surface.

The neuroendocrine substrate underlying seasonal reproduction in birds involves at least three components (Follet 1984), (1) encephalic photoreceptors that are coupled to a biological clock to measure day length, (2) the gonadotropin-releasing hormone (GnRH) system and ancillary neural pathways that impinge on it, and (3) the peripheral endocrine system. Recently it was suggested that the extraretinal photoreceptor in the hypothalamus is essential for seasonal reproduction (Yoshikawa and Oishi, 1998). The retinal protein in the cerebral ganglion found in the present work is a candidate for the photoreceptor for light-induced spawning of ascidians.

Gonadotropin-releasing hormone (GnRH) of the vertebrate brain, which has originally been identified as a hypophysiotropic hormone, is believed to act also as a neuromodulator (Jones *et al*., 1984, Oka and Matsusita, 1993). In addition to the GnRH found in seven classes of vertebrates, the two new GnRH family members have been identified in an ascidian (Powell *et al*., 1996, Craig *et al*., 1997). Recently, the morphology of the GnRH neuronal system of the ascidian, *Ciona intestinalis*, was examined by immunocytochemistry and showed that GnRH immunoreactive neurons exist on the surface of the cerebral ganglion (Mackie 1995, Tsutsui *et al*., 1998). A major population of GnRH neurons were distributed in a sheet-like fashion on the ventral side of the posterior half of the cerebral ganglion, adjacent to the border between the cerebral ganglion, and the neural gland. A minor population was located on the right half of the posterodorsal surface of the ganglion, surrounding the right posterior nerve root. GnRH immunoreactive neurons on the ganglion appeared to exist mainly outside the cellular cortex. GnRH immunoreactive cells run from the cerebral ganglion towards the gonads along the dorsal strand and gonoducts. Though the distribution of GnRH immunoreactive cells on the cerebral ganglion in *Halocynthia roretzi* in the present work was slightly different from that of *Ciona intestinalis* (Terakado, pers. com.; Ohkuma, unpublished), GnRH immunoreactive neurons on the ganglion in *Halocynthia roretzi* i appeared to exist outside

the peripheral cellular cortex where retinal proteins were located in the present work.

GnRH cells showed spontaneous regular discharges, which are dependent on their endogenous pacemaker properties (Oka, 1997) Thus, it is expected that GnRH neurons play a central role in the reproduction clock. Recently, electrophysiological evidences have suggested that the cerebral ganglion of adult ascidian is a light-sensitive organ. Spontaneous regular discharges at the ventral side of the cerebral ganglion of adult ascidians were decreased upon light stimuli for *Ciona savignyi* using intracellular recording (Tsutsui and Oka, in press) and *Halocynthia roretzi* using extracellular recording (Katagiri, pers. com.). In conjunction with the fact that close localization between retinal protein bearing cells and GnRH bearing cells, it is suggested that a retinal protein is a candidate for triggering biological clock of reproduction.

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