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# An Improved Photoelectric Method for Recording Motile Responses of Individual Leucophores

Ryozo Fujii*, Takashi Yamada[1], Tomohiro Fujita and Noriko Oshima

*Department of Biomolecular Science, Faculty of Science, Toho University,
Miyama, Funabashi, Chiba 274, Japan*

**ABSTRACT**—An improved photoelectric method for recording motile responses of individual leucophores was developed. Leucophores from scales of the medaka, *Oryzias latipes*, were used as a model. The intensity of light scattering from the peripheral dendritic zone ($S_p$) and that from the cell body ($S_c$) of each leucophore were separately transduced to the current changes, which were then converted to voltages, and appropriately amplified. Output voltages of both channels were represented as "$V_p = A_1 \times S_p$", and "$V_c = A_2 \times S_c$", where $A_1$ and $A_2$ were the overall amplification coefficients for these channels, respectively. When leucosomes dispersed from the cell body into the dendrites, the $V_p$ increased and the $V_c$ decreased, while the reverse changes occurred when the leucosomes aggregated. Using a simple electronic circuit, the remainder of the two outputs, namely, $V_p - V_c$, could easily be obtained and recorded. As alternative expressions of the response, the ratio of the outputs ($V_p/V_c$), its square root ($\sqrt{V_p/V_c}$), or $\sqrt{(V_p + v_p)/(V_c + v_c)}$ could also be recorded using integrated circuits developed for such purposes ($v_p$, $v_c$: voltage biases applied to $V_p$ and $V_c$). The relative merits of these types of analysis are discussed. Similar analysis should also be applicable to the motile activities of iridophores of the dendritic type, such as those of some gobiid fish.

## INTRODUCTION

Leucophores, which contain light-scattering organelles, known as leucosomes, are the chromatophores which exist in the dermis of some teleostean species (Fujii, 1993). Like other chromatophores of the dendritic type, they show motile responsiveness to various stimulatory cues. Because of their optical properties however, they are the most difficult chromatophores to quantitatively assess for their responses. Sometimes for example, part of the length of a dendritic process filled with the leucosomes was measured (Kinosita, 1953, 1963), while other studies have measured the diameter of the domain occupied by the leucosomes (Miyoshi, 1952). Some workers have described leucophore responses using the so-called "melanophore index" ("MI") that was originally devised for recording the motile responses of melanophores of the clawed toad, *Xenopus laevis* (Hogben and Slome, 1931). Since the same method can also be adapted for assessing motile activities of various chromatophores of the dendritic type, the method may appropriately be designated as the "chromatophore index", or "CI" (Fujii, 1969, 1993). When applied to leucophores, the method may appropriately be called the "leucophore index", or "LI". Photomicrographs taken in series usually provide records of a higher quality and with more in-

formation (Menter *et al.*, 1979; Obika, 1988). However, such methods cannot follow rapid responses of the cells, and the results must be presented with only a limited number of images. More recently, video-tape recording of the microscopic images has been developed as an even more convenient method (Fujii *et al.*, 1991a), but this still suffers from lack of resolving power, and as with photomicrography, only selected images can be conveniently published.

Meanwhile, a photoelectric method has become more widely employed for recording the motile responses of chromatophores. In brief, the method measures the increases or decreases in light transmitted through a small area of the skin within the visual field of a common light microscope. Because of its easier applicability to chromatophores with heavily pigmented organelles, melanophores have naturally been studied frequently with this method (Fujii, 1959; Oshima and Fujii, 1984). This method has sometimes been applied to other light-absorbing chromatophores, such as xanthophores of the medaka *Oryzias latipes* (Oshima and Fujii, 1985) and erythrophores of some xiphophorine and tetra fishes (Oshima and Fujii, 1985; Hayashi *et al.*, 1993; Murata and Fujii, 1995). We have recently used this method to study the novel blue chromatophores (cyanophores) of a callionymid fish, *Synchiropus splendidus* (Goda and Fujii, 1995). The photoelectric method allows us to continuously measure the motile response of even a single chromatophore.

With a partial modification of the technique for measuring the light scattered from the cell, Fujii and Miyashita (1979) attempted to record the motile response of a leucophore of

* Corresponding author: Tel.   +81-474-72-7518;
            FAX. +81-474-75-1855.
[1] Present address: Diagnostics Research Laboratories, Fujirebio, Inc., Komiya-cho, Hachioji, Tokyo 192, Japan.

the medaka, *Oryzias latipes*. To obtain reproducible results however, scattering from the aggregate of leucosomes in the perikaryon had to be hindered. In other words, the recorded data were due to changes in light scattering only from peripheral, dendritic portions of the cell. In order to measure responses from the entire leucophore, we have now designed a more plausible and reliable method, and this paper reports this improved technique and reports some preliminary examples of its use.

## MATERIALS AND METHODS

### Materials

Adult individuals either of the wild type or of the orange-red variety of the medaka, *Oryzias latipes*, were used in this study, and leucophores within scales were examined for their motile responses. Motile iridophores on the scales of the dark sleeper goby, *Odontobutis obscura obscura*, were also tested for the possible application of this method, because, since they are dendritic, these cells show cellular motility similar to the leucophores (Iga and Matsuno, 1986; Fujii *et al.*, 1991b). The medakas and the gobies were obtained from local dealers, and before all experiments, the fish were kept in freshwater aquariums at least for a few days for acclimatization.

With either species of fish, scales were plucked from the anterior, dorso-lateral part of the trunk, and immersed in a physiological saline solution for teleosts which had the following composition: (in mM): NaCl, 125.3; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 1.8; D-(+)-glucose, 5.6; Tris-HCl buffer, 5.0 (pH 7.3). By means of a fine glass needle (diameter: ca. 0.4 mm) glued at both ends to the coverslip, a scale plucked from a medaka or goby was held with its bony scale side in contact with the coverslip. The method of irrigating the scale with physiological saline or with stimulant solutions in a small perfusion chamber was essentially identical to that described elsewhere (Fujii and Miyashita, 1975; Oshima and Fujii, 1984).

### Optical system

In preliminary observations, an Olympus dark field epi-illumination microscope (Neopak NJTr, Olympus, Tokyo) was used. For later quantitative measurements of cellular responses, a Nikon industrial light microscope with similar performance (Optiphot XT-BD, with CF-BD Plan objective lenses; Nikon, Tokyo) was employed. Since they were installed with transmission and incident-light illumination systems, these microscopes enabled us to observe the chromatophores either by usual transmission optics or by incident light illumination, or by both. Thus, a comparison of the images of the cells by both optical systems could easily be made. We used a Nikon trinocular assembly of the T type in order to photoelectrically measure the motile response while observing the cell through binocular eye-pieces.

### Photosensing

The differential photosensing part of the system developed in this study is schematically displayed in Fig. 1. Small-sized, high quality silicon photodiodes (S1226-18BK, Hamamatsu Photonics, Hamamatsu) were conveniently adopted as the photoelectric transducers, and were installed inside the photographic column of the trinocular assembly. On the rear focal plane of the objective lens, upon which the real image of the object was projected, a finely ground thin glass plate was placed to homogeneously diffuse the light-rays to the photosensors. The concentric separation of the light beams was also performed on this plane. For recording the light scattering from the cell body ($S_c$), a cut end of a plastic optical fiber (0.5 mm diameter) was set just in contact with the center of the ground surface of the glass plate. The optical fiber was sheathed within a thin stainless steel tube with an inner and outer diameter of 0.5 and 0.8 mm, respectively. The other end of the fiber was attached to a silicon photo-
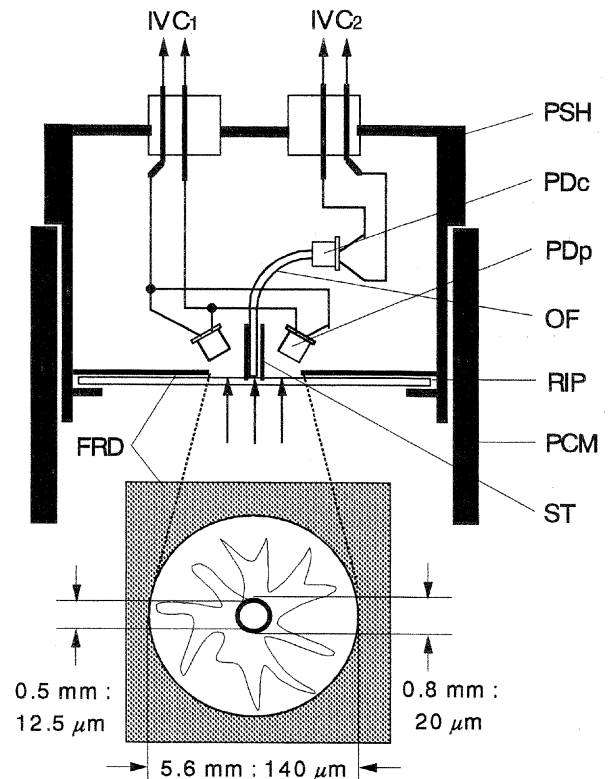


**Fig. 1.** Diagram showing the light sensing components of the system for quantitative recording of the response of a single light-scattering dendritic chromatophore. The apparatus was designed for independently measuring lightrays scattered from the central part and from the peripheral part of the cell independently. At the top of the figure, is shown the arrangement of the photodiodes within their housing (PSH) adapted on the photographic column (PCM) of the trinocular assembly of the microscope. At the bottom, the dimensions of the field-restricting diaphragm (FRD) with a projected image of a leucophore are displayed. $IVC_1$: current-to-voltage converter of channel 1, $IVC_2$: current-to-voltage converter of channel 2, OF: optical fiber, $PD_C$: photodiode for sensing light scattering from central part of the cell, $PD_P$: photodiode for sensing light scattering from peripheral part of the cell, RIP: real image plane, or rear focal plane of the objective lens, ST: stainless steel tubing.

diode. Using the 40 x objective lens, we were thus measuring $S_c$ from a central circular area 12.5 μm in diameter, which approximated the size of the cell body of a single chromatophore (Fig. 1).

In order to measure the scattering of light from the peripheral, dendritic zone of the leucophore ($S_p$), the light-rays hitting a circular area 5.6 mm in diameter on the plane of the real image were detected (Fig. 1). By design, the concentric central zone of 0.8 mm in diameter was occupied by the stainless steel tubing, and thus, the light scattering from the zone between two concentric circles with the diameters of 5.6 and 0.8 mm could be measured. Using the 40 x objective lens, the actual area to be measured on the skin was within the circular area of 140 μm in diameter, excluding the central circular zone 20 μm in diameter (Fig. 1). Usually, the domain occupied by a leucophore from *Oryzias* or by a motile iridophore from *Odontobutis* was within the 140 μm diameter. For an accurate measurement of light scattering from this area, three photodiodes were placed at equal distances from each other, namely, on the apexes of an equilateral triangle.

An eye-piece micrometer with an inscribed lattice pattern could be used to adjust the position of the cell body of the chromatophore

with the mechanical stage adapted to the microscope stage. However, it proved to be more practical to position the cell body at the center of the domain by monitoring the output voltage of the channel to measure the light scattering from the cell body (cf. next section). Such a procedure could be done rather easily, because, when equilibrated in physiological saline, light-scattering organelles in the leucophore were normally aggregated in the cell body.

## Electronic circuits

Simplified diagrams of the electronic division of the apparatus designed for processing output currents of the photosensors are shown in Fig. 2. Coupled with the photosensing system described above, these circuits enabled us to measure $S_p$ and $S_c$ differentially and continuously.

Photoelectric current from either of the two channels, namely, from the photosensor for $S_c$, or from the assembly of photosensors for $S_p$, was converted separately into voltage change by a current-to-voltage converter using a high quality operational amplifier (IVC, Fig. 2A). In this design, we adopted a MAX421 (CPD-type package; Maxim Integrated Products, Sunnyvale, CA) as the appropriate operational amplifier. This was so designed that the input offset was automatically compensated with the built-in chopper-stabilized mechanism at 400 Hz, in addition to the desirable feature of very high input impedance ($10^{12}$ $\Omega$). The efficiency of the conversion was modified by adjusting the value of the feedback resistance ($Rf_1$, $Rf_3$) of the operational amplifiers. The voltage outputs were further amplified by means of one-step direct-current amplifiers of the non-inverting type. Again, identical operational amplifiers to those employed in the preceding step were adopted (DCA, Fig. 2A), and again, the amplification was adjusted by changing the feedback resistance ($Rf_2$, $Rf_4$) of the operational amplifiers.

In either channel, the final voltage output ($V_p$ or $V_c$) of the circuits can be expressed as the product of the intensity of light scattering ($S$), the efficiency of the current-to-voltage conversion, and the amplification factor of the direct-current amplifier. If the product of latter two are designated as $A_1$ or $A_2$, as the overall amplification factor, the final voltage output of each channel can be described as: $V_p = A_1 \times S_p$, or $V_c = A_2 \times S_c$.

When leucosomes disperse from the cell body into dendrites, $V_p$ increases and $V_c$ decreases, while the reverse changes occur when the leucosomes aggregate. If we desired, these changes can be separately registered on a chart recorder having more than two channels. During the actual recording of the cellular response, however, these changes are more conveniently integrated into a single channel and recorded.

$A_1$ or $A_2$ can be adjusted independently throughout a wide range by adjusting the efficiency of the current-to-voltage converter and/or the amplification factor of the direct current amplifier that follows the converter (Fig. 2A). Appropriate values for $A_1$ and $A_2$ were empirically determined beforehand on cells with average sizes and optical characteristics. Since the contribution of the central part to the overall change of light scattering was less than that of the peripheral zone, the range of the net voltage changes of channel 2 was adjusted to 30 − 50% of that for the peripheral zone.

The output voltages of the two channels, $V_p$ and $V_c$, were then processed in three different ways. First, the difference between $V_p$ and $V_c$ was determined (Fig. 2B). $V_c$ was first converted to $-V_c$ using a polarity reversing circuit. $V_p$ and $-V_c$ were then summed by an adding circuit executed by the operational amplifier of the same type as that described above (MAX421), and recorded on a paper-chart recorder (EPR-10B, or EPR-221, Toa Electronics, Tokyo). Their ratio, $V_p/V_c$, was calculated continuously by an analog integrated circuit, and was recorded (Fig. 2C). For this purpose, an item especially designed for calculating the division of two input signals (AD535JH, Analog Devices, Tokyo) was adopted. Finally, the $V_p/V_c$ ratio was fed to another integrated circuit (AD533JH, Analog Devices), from which the square root of the input voltage could be obtained. The result,
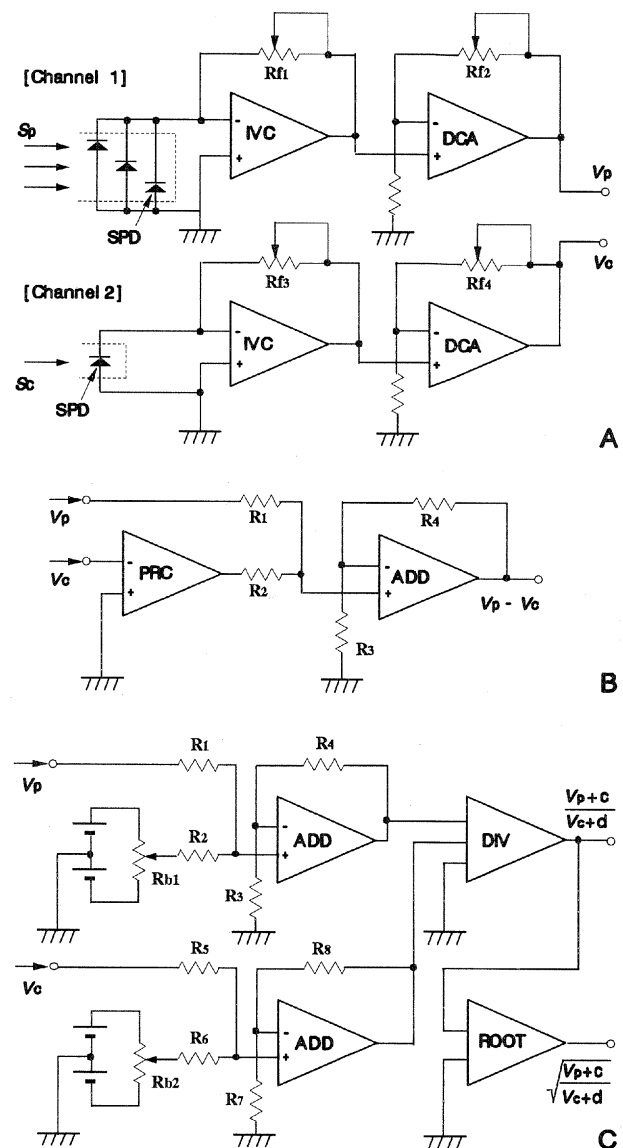


**Fig. 2.** Diagrams of the electronic processing parts employed. (**A**) Common initial steps consisting of two sets of serial combinations of current-to-voltage converters (IVC) and direct-current amplifiers of the non-inverting type (DCA). Channel 1 was for obtaining $V_p$, while the channel 2 was for $V_c$. $Rf_1$ - $Rf_4$: feedback resistors for IVC or DCA, SPD: silicon photodiode. (**B**) Adding circuit (ADD) for obtaining the sum of the output voltage of channel 1 ($V_p$) and the negative value of the output voltage of channel 2 ($-V_c$), namely, $V_p - V_c$. The negative value was obtained by means of a polarity-reversing circuit (PRC). $R_1$ − $R_4$: fixed resistors; in the present series of measurement, we adopted the same value (10 k$\Omega$) for them. (**C**) Circuits for the 2nd and the 3rd treatments in the present series of measurement as desribed in the Results section. By means of adding circuits (ADD), appropriate voltage biases could be independently applied to $V_p$ and $V_c$. The results were fed to the divider circuit (DIV) for obtaining their division (2nd treatment). In the 3rd treatment, the output voltage of the divider was further introduced to a "rooter" circuit (ROOT) to extract its square root. In the present trial, the same value (10 k$\Omega$) was employed for $R_1$ − $R_4$ and also for $R_5$ − $R_8$. Rb$_1$, Rb$_2$: variable resistors giving appropriate voltage biases to adding circuits. For further details, see text.

$\sqrt{V_p/V_c}$, was routed to the paper-chart recorder (Fig. 2C).

The polarity reversing device was put at the final stage of the electronic division of the present system, although not shown in Fig. 2, in order to reverse the polarity of the output signals to the paper-chart recorder. Usually, it was set so as to record the progress of the dispersion of light-scattering organelles in the cell as the upward shift of the trace on the chart.

Since the scattering of light from part of a cell was weak, a considerably higher amplification of the signals was needed in either channel. Even though the utmost care in constructing and wiring the circuits had been taken, some noise was inevitably introduced in this system. In order to decrease this noise, an active, low-pass filter was placed between the output of the system and the recorder (not shown in Fig. 2). A second-order Butterworth type circuit was made, which was installed with the part for altering the cutting frequency stepwisely. A cutting frequency of 0.5 Hz was found to yield good results without noticeable deformation of the shape of the cellular response.

### Computer graphics for analyses

In order to examine which of the three treatments mentioned above for integrating the two output signals would be more appropriate, computer graphics were ued, in addition to the actual empirical trials on the cells. A program for the simulation was developed using $N_{88}$-DISK BASIC(86), by which changes could be observed in the form of multiple-ordered algebraic functions by altering the values of any coefficient in the function. In fact, the virtual relationship of the input signals to an integrated output could easily be visualized by changing various parameters of the functions on the display of a personal computer.

### Electrical stimulation

In some experiments, the chromatophores were stimulated electrically to induce the dispersion of organelles. The method was fundamentally identical to that employed in earlier studies using isolated fin pieces (Fujii and Novales, 1968, 1969; Fujii and Miyashita, 1975), but modified somewhat for application to the scales. An electronic stimulator (SEN-3201, Nihon Kohden, Tokyo) was used to stimulate nerves controlling the chromatophores. A stimulating electrode, a Pt wire 500 μm in diameter and insulated except at the cut end, was gently placed around the rostral margin of the piece of skin attached on an isolated scale. A leucophore or a motile iridophore located about 500 μm posterior to the electrode was measured for the cellular response. The polarity of the stimulating pulses was set to be negative in reference to this electrode. Another Pt wire dipped in the bottom of the perfusing chamber served as an indifferent electrode. Rectangular pulses of 1-msec duration and 10.0 V in strength were applied through the stimulating electrode at various frequencies. In order to reduce polarization at the surface of the electrodes, the pulses were biphasically modified by inserting a condenser (0.1 μF) between the output of the stimulator and the stimulating electrode. A storage oscilloscope (5111A, Tektronix, Beaverton, Oregon) was used for monitoring the stimulating pulses.

### Chemical stimulation

Norepinephrine (hydrochloride salt; racemic modification; Sankyo, Tokyo) was used to induce the dispersion of the organelles either in leucophores or in motile iridophores. The concentration of the amine was given in terms of the physiologically active L-(–)-isomer. As the sympathetic neurotransmitter, norepinephrine has been shown to aggregate chromatosomes in common light-absorbing chromatophores via activation of α-adrenoceptors (Fujii, 1993; Fujii and Miyashita, 1975; Fujii and Oshima, 1986). In *Oryzias* leucophores, however, the dispersion of leucosomes in response to sympathetic stimuli is mediated through adrenoceptors of the β type (Obika, 1976; Iga *et al.*, 1977). By contrast, the reflecting platelets in the motile iridophores of the dark sleeper have been shown to disperse through the mediation of α-adrenoceptors (Iga *et al.*, 1987).

In some experiments, a K⁺-rich saline solution was employed to stimulate the chromatophore, because an elevated concentration of K⁺ ions is known to act as a sympathetic stimulus via the release of adrenergic transmitter from the postganglionic fibers (Fujii, 1959, 1993; Fujii and Oshima, 1986). In some preliminary observations, such as those shown in Fig. 1, a K⁺-rich saline in which Na⁺ ions in the primary saline were totally replaced with equimolar concentration of K⁺ ions was employed. In later studies made using the photoelectric method, a saline containing 50 mM K⁺ was exclusively used, in which the concentration of Na⁺ ions was also compensatorily decreased so that the final osmolarity was the same as that of the standard saline solution.

All the physiological measurements were carried out at a room temperature between 20 and 25°C.

## RESULTS

When equilibrated in physiological saline, melanosomes in the melanophores of the medaka scale were in a dispersed state, as in many other common teleosteans (Fig. 3A). In contrast, under the same condition, leucosomes in the leucophores were tightly aggregated in the perikarya (Fig. 3B, C). It is known that various stimulatory cues that cause aggregation of pigment in the melanophores disperse leucosomes. In the serial photomicrographs, the leucosome-dispersing effect of increased K⁺ concentration could be demonstrated (Fig. 3D, E, F). In this particular series, incidentally, we employed a K⁺-rich saline in which Na⁺ ions in the primary saline were totally replaced with equimolar concentration of K⁺ ions. It should be noted here that the light scattering from the mass of aggregated leucosomes was fairly large (Fig. 3C, D), which resulted in considerable difficulty in photoelectrically measuring the responses of leucophores, thus prompting us to improve the apparatuses.

### The first treatment

The sequence of the motile responses of leucophores is shown in Fig. 3. When we used the microscopic system coupled with the electronic processing parts as shown in Fig. 2A, the following events took place: When leucosomes dispersed from the cell body into dendrites of a leucophore, the overall output voltage of channel 1 ($V_p$) increased, while that of channel 2 ($V_c$) decreased (Fig. 4). The reverse changes occurred when the leucosomes aggregated. If we assume the progress of the responses to be linear for the sake of simplicity, the output voltages of the two channels can be expressed as:

$$V_p = ax + b \quad \text{------------------------------------------------ (1)}$$
$$V_c = -cx + d \quad \text{----------------------------------------------- (2)}$$

Here, $x$ stands for the extent of the leucosome-dispersing response, which is scaled on the abscissa of Fig. 4. In the present simulation, we define that $x$ changes within the range of 0 to 1. Here, 0 corresponds to the state in which leucosomes are completely aggregated within the perikaryon, while 1 represents the state when they are dispersed throughout the cytoplasm. The increase in the value of $V_p$ and/or the decrease in $V_c$ denote the progress of the leucosome-dispersing response, whereas their reverse changes indicate the progress of the leucosome-aggregating response. In the present treat-
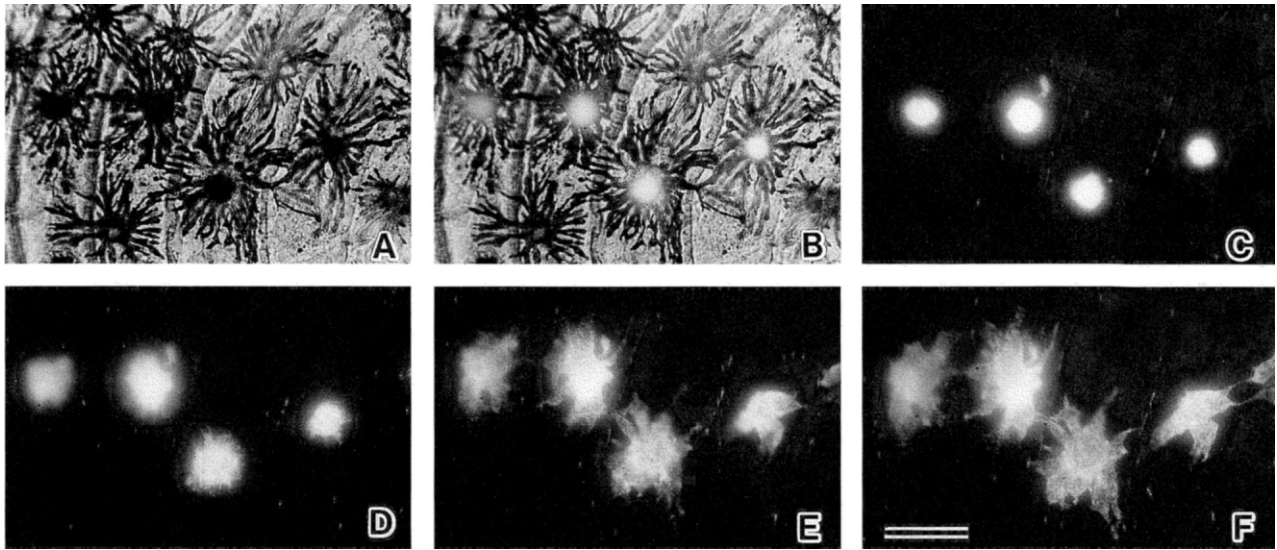
**Fig. 3.** Serial photomicrographs showing the motile responses of leucophores on an isolated scale of a wild-type medaka, *Oryzias latipes*. The scale is viewed from the dermal side. (**A**) Equilibrated in physiological saline. Ordinary transmission optics. Melanophores with fully dispersed melanosomes are seen. (**B**) The same state, but both transmission and dark-field epi-illuminations were employed for observing the melanophores and the leucophores simultaneously. Four bright, light-scattering masses are the aggregates of leucosomes in the perikarya of leucophores. (**C**) The same state, but viewed under epi-illumination only. This type of illumination was employed for **D-F**. (**D**) Seventy sec after application of $K^+$-rich saline; leucosomes in the leucophores have begun to disperse into the dendrites. (**E**) Three min after application of $K^+$-rich saline; the medium was changed to normal saline solution, and after 60 sec perfusion, leucosome-dispersion response was still proceeding. (**F**) Four min thereafter, and leucosomes are almost completely dispersed. Scale bar: 100 μm.

ment, both $V_p$ and $V_c$ are also defined to change between 0 and 1 on the $y$ axis or the ordinate of Fig. 4.

In the simulation displayed in Fig. 4, values for a, b, c and d in equations "1" and "2", were set to be positive for easier analysis. As mentioned above, the actual change of the light-scattering from the central region is usually smaller than that from the peripheral part of the cell. Based on a rough estimation of the light-reflecting properties of the photomicrographic images of leucophores, we recognized that the contribution of the former was usually less than half that of the latter. In the simulation exhibited in Fig. 4, therefore, we adopted the value of 40% for this. Since both $V_p$ and $V_c$ are linear functions of $x$ within the same region, the incline of $V_c$, namely c, was thus set to be 40% that of $V_p$, namely a. As practical values for a and c, we used 0.50 and 0.20, respectively. In consideration of the actual situation during the measurement of the response of leucophores, 0.07 and 0.35 were representatively given to b and d, respectively. The two lines denoted as A and B in Fig. 4 are those generated when these values are substituted in equations "1" and "2".

When the response of a leucophore proceeds, either in the leucosome-dispersing or the aggregating direction, $V_p$ and $V_c$ show an inverse correlation with each other, as described above. Thus, the integrated response ($y_I$) may be simply expressed as the remainder of these two output voltages as:

$$y_I = V_p - V_c = (a + c) x + b - d \quad \text{------------------------------} (3)$$

As one popular application, an operational amplifier could be employed as the adding circuit, and such a treatment could easily be performed, as described in the Materials and Meth-
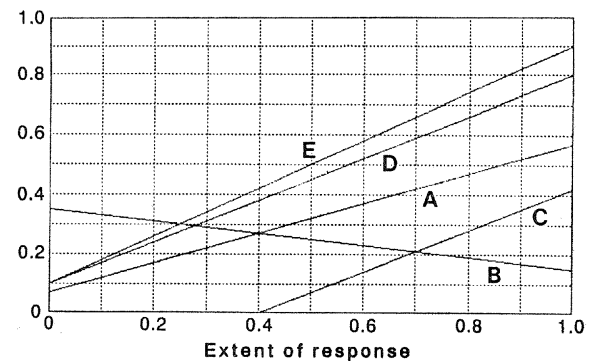


**Fig. 4.** Simulated lines by computer graphics for examining the appropriateness of the method described in the 1st treatment described in the Results. A: Postulated change of voltage output of the initial step for channel 1 ($V_p$). B: Postulated change of voltage output of the initial step for channel 2 ($V_c$). C: Line for $y_I$ ($= V_p - V_c$). D: Line shifted parallel from $y_I$, in order to position the pen around 0.1 within the whole span of the recording chart. E: Line changed of its inclination from line D in order to include the whole range of the cellular response within the range of ca. 0.1 - 0.9 on the recording chart. Abscissa ($x$): State of leucophore, or the extent of leucosome-dispersing response, expressed as the value between 0 and 1.0, where 0 stands for the state in which leucosomes are completely aggregated in the perikaryon, while 1.0 indicates the state in which leucosomes are completely dispersed in the cytoplasm. Ordinate for lines A and B ($y$): Changes of $V_p$, $V_c$, $V_p - V_c$, and the modified functions (Region: 0 − 1.0). In this particular exhibition, we set $V_p = 0.5x + 0.07$, and $V_c = -0.2x + 0.35$. For further explanations, see text.
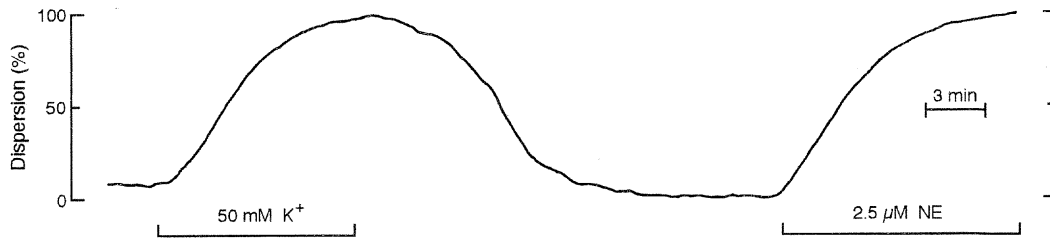
**Fig. 5.** Typical recording of the response of a leucophore on a medaka scale, *Oryzias latipes*, to an elevated concentration (50 mM) of K$^+$ ions in the perfusing medium. The recording was made employing the 1st treatment described in the Results. Finally, 2.5 µM norepinephrine solution was applied to bring about the full level of leucosome dispersion.

ods section (see also Fig. 2B). Line C in Fig. 4 indicates this relationship. By adjusting the position of the pen (Fig. 4, line D), and by changing the sensitivity of the recorder, the range of the leucophore response can be recorded within the desirable region on the recording chart (Fig. 4, line E). In this treatment, the recorded voltage change is a first degree equation. Without need of complicated decoding processes, therefore, further analyses can easily be performed as necessary.

As a typical recording using this type of analysis, Fig. 5 shows the responses of a leucophore from medaka to an increase in K$^+$ concentration and then to norepinephrine. The dispersion of leucosomes in response to these stimuli are clearly observable.

## The second treatment

As a function of integrating the changes in the light scattering both from the central and the peripheral parts of the leucophore, we then considered their ratio:

$$y_{II} = V_p/V_c = (ax + b)/(-cx + d) \tag{4}$$

With the same values for a–d, the lines A and B in Fig. 6 indicate the same functions as those shown in Fig. 4. The obtained relation took a concave form, shown as curve C in the figure. The equation indicates that $y_{II}$ diverges towards
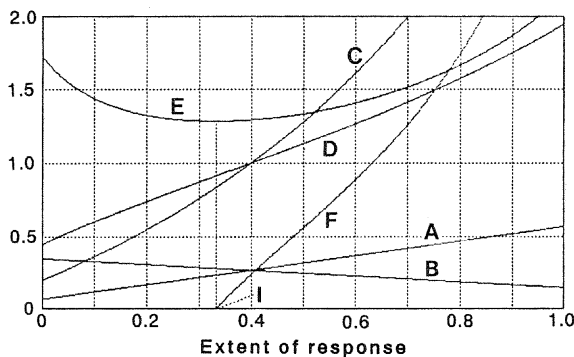


**Fig. 6.** Simulation for examining appropriateness of the methods described in the 2nd and the 3rd treatments described in the Results section. A: Postulated change of $V_p$. B: Postulated change of $V_c$. C: Curve for $y_{II}$ ( $= V_p/V_c$). D: Curve for $y_{III}$ ( $= \sqrt{V_p/V_c}$). E: Curve for $dy_{III}/dx$. F: Curve for $d^2y_{III}/dx^2$. In this particular exhibition, too, we set $V_p = 0.5x + 0.07$, and $V_c = -0.2x + 0.35$. The explanations given in the legend for Fig. 4 regarding the abscissa and ordinate also apply to this figure. For further explanations, see text.

positive infinit when $x$ approaches d/c. In practice, such a condition is unlikely, since it corresponds to the state in which the light scattering from the central part of the cell is completely lost. The function approaches 0 when $x$ approaches –b/a, but, as described above, negative values for a or b were out of the definition.

The first order differential function of equation "4" is calculated as:

$$dy_{II}/dx = (ad + bc)(-cx + d)^{-2} \tag{5}$$

When $x = $ d/c, the equation diverges towards infinit, but such a condition is not practical within the defined region of $x$.

From equation "5", we can obtain the second order differential function of the relation as:

$$d^2y_{II}/dx^2 = 2c(ad + bc)(-cx + d)^{-3} \tag{6}$$

Under the present condition, $x$ is smaller than d/c, and the function is positive. In other words, the incline of equation "4" increases within the defined region ($0 \leqq x \leqq 1$).

## The third treatment

Since function "4" took a concave shape, we concluded that the relation could not be employed as such. We thus tried to analyze changes in the shape of the function in which function "4" was further extracted to its square root, as:

$$y_{III} = \sqrt{V_p/V_c} = \sqrt{(ax + b)/(-cx + d)} \tag{7}$$

The function is shown as curve D in Fig. 6. In order that the value within the root be positive, $x$ must be within the range between –b/a and d/c. Such conditions are naturally satisfied.

The first and the second order derivatives of equation "7" were calculated as:

$$dy_{III}/dx = (1/2)(ad + bc)(ax + b)^{-1/2}(-cx + d)^{-3/2} \tag{8}$$

$$d^2y_{III}/dx^2 = (1/4)(ad + bc)(ax + b)^{-3/2}(-cx + d)^{-5/2}$$
$$(4acx + 3bc - ad) \tag{9}$$

Curves E and F in Fig. 6 show functions (8) and (9), respectively. Examination of the product members constituting these equations shows that all the terms except for ($4acx + 3bc - $ ad) are positive within the defined region. On the other hand, the points of inflection of the initial function "7" can be obtained when its second-order differential of the initial function equals zero. Thus, the point can be obtained the point by setting the last term of equation "9" to be zero, the point thus obtained being $x = $ (ad $-$ 3bc)/4ac.

This means that the term ($4acx + 3bc - $ ad) is positive or negative, when $x$ is larger or smaller than (ad $-$ 3bc)/4ac, re-

spectively. Within the region where $x$ is smaller or larger than the point, the curve takes a convex or concave shape, respectively, and approximates a straight line in the vicinity of the inflection. Substituting various positive numbers for a, b, c and d into equations "1" and "2", we examined changes in the shapes of curves of the function as well as of its derivatives. In this way, we could recognize that function "7" becomes straighter as − b/a becomes more negative and/or that d/c becomes more remote from the upper limit of the actual range, namely, unity.

## Modification of the 2nd and 3rd treatments

If we want to have the point of inflection around the middle way of the response ($x = 0.5$), we can do so. In the representative simulation displayed in Fig. 4 and also in Fig. 6, we have already adopted 0.50, 0.07, 0.20 and 0.35 for a, b, c and d, respectively. We can perform the operation, for example, by changing only d. At the point of inflection, the term "4acx + 3bc − ad" should equal zero, as mentioned above. Thus, d = 4cx + 3bc/a. Substituting real numbers into the equation, we can obtain the value of d as 0.484. Changing the value of d means shifting function "2". By applying a voltage bias to the input for channel 2 of the divider circuit, such a shift can easily be performed electronically. For this purpose, we employed a simple circuit shown as $Rb_2$ in Fig. 2C. If such precautions are duly taken before recording the leucophore response, the method may be convenient, although such precise and sensitive settings are not needed for practical purposes.

In order to improve the linearity in recording the cellular response, we have tried to further modify the method. Observations on the changes of curves for function "7" and for its derivatives ("8", "9"), showed that when the intercepts of the original functions ("1" and "2") became larger, the linearity of the integrated relation ("7") increased. In the simulation shown in Fig. 7, the intercepts of functions "1" and "2" were increased by 1.0, namely, b = 1.07 and d = 1.35 (cf. lines A ($Y_p$) and B ($Y_c$)). Looking at the actual relation and its derivatives (lines D, E and F in Fig. 7), we recognized that the linearity was significantly improved if compared with the case of $y_{III}$. Such
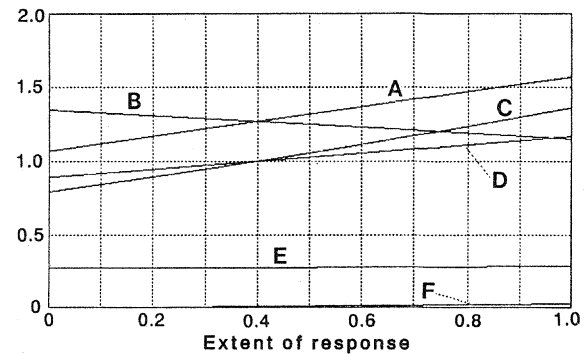


**Fig. 7.**  Simulated lines and curves for improving the 3rd treatment described in the Results section. In this particular simulation, either $V_p$ or $V_c$ was shifted up by 1. A and B: Line for $Y_p = V_p + 1 = 0.5x + 1.07$. B: Line for $Y_c = V_c + 1 = − 0.2x + 1.35$. C: Curve for $Y_p/Y_c$. D: Curve for $\sqrt{Y_p/Y_c}$. E: Curve for $d/dx\,(\sqrt{Y_p/Y_c})$. F: Curve for $d^2/dx^2(\sqrt{Y_p/Y_c})$. The explanations given in the legend for Fig. 4 regarding the abscissa and ordinate also apply to this figure. For further explanations, see text.

shifts of the relations can be easily realized by adding voltage biases to the input of the divider circuit. By adjusting $Rb_1$ and/or $Rb_2$, such operation can be easily done electronically.

As a typical recording obtained by this method, Fig. 8 illustrates the responses of a leucophore from a medaka scale to electrical stimulation. Leucosomes in the cell dispersed in response to pulses of various frequencies. In this way, practically identical recordings to those obtained by the method described as the first treatment could also be obtained by this method.
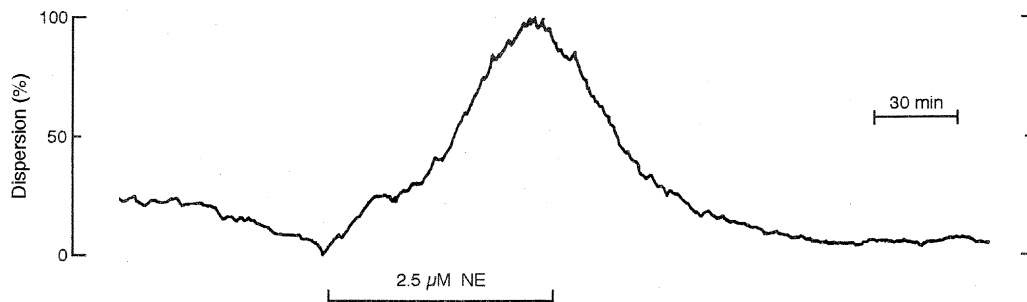
## Application to motile iridophores

We then applied the developed method to motile iridophores of the dark sleeper goby, *Odontobutis obscura obscura*. Figure 9 shows the typical responses of a cell to norepinephrine recorded while the apparatus was set to operate under conditions of the first treatment. The upward shift of the trace was due to the dispersion of light-reflecting platelets into the peripheral dendritic zone, while the downward
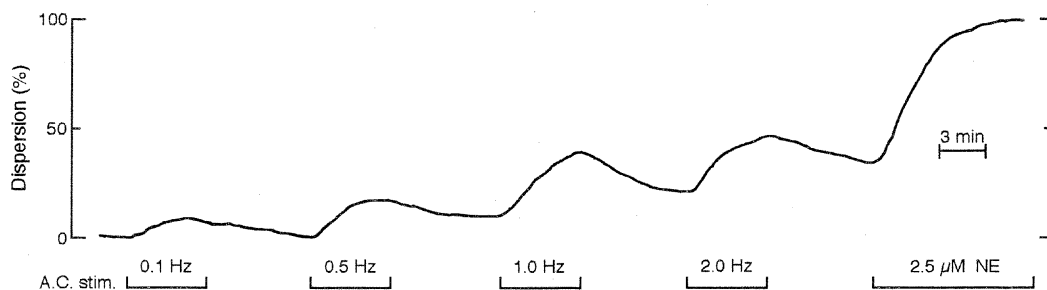


**Fig. 8.**  Typical recording of the responses of a leucophore of the medaka, *Oryzias latipes*, to electrical nervous stimulation of chromatic nerves and to norepinephrine. In this recording, the amounts of light scattering from the dendritic zone ($S_p$) and from the central body ($S_c$) were processed by the 3rd treatment, as described in the Results. The stimulation was performed by biphasically deformed square pulses (1 msec, 10.0 V) at the various frequencies noted in the figure. Finally, 2.5 μM norepinephrine solution was added to stimulate a maximal level of leucosome dispersion.

**Fig. 9.** Typical recording of the response of a motile iridophore on a scale of the dark sleeper goby, *Odontobutis obscura obscura*. After equilibration in physiological saline, 2.5 µM norepinephrine (NE) was applied. The recording was made using the electronic circuit for the 1st treatment described in the Results. A gradual dispersion of light-reflecting platelets took place. Upon rinsing the scale with physiological saline again, reaggregation of them occurred.

shift indicates their aggregation into the cell body. In describing the optical property of motile iridophores, incidentally, the term "light scattering" as used for leucophores may appropriately be replaced with "light reflectance" because the lightrays are reflected mainly from the flat surfaces of the reflecting platelets (Fujii *et al.*, 1991b; Fujii, 1993).

## DISCUSSION

In the skin of teleosts, light-scattering and/or reflecting chromatophores usually coexist with light-absorbing chromatophores. For example, in the medaka, leucophores are present along with melanophores and xanthophores. In the orange-red variety of the species, melanophores are replaced with colorless amelanotic melanophores (Fujii, 1993). The motile iridophores of the dark sleeper goby, another experimental material used in this study, also exist with melanophores and xanthophores.

It should also be noted that leucophores of the medaka are normally present just under melanophores concentrically (Miyoshi, 1952; Obika, 1988; Fujii and Miyashita, 1979). Therefore, unless a leucophore happens to exist solitarily, it is practically impossible to measure responses of leucophores photoelectrically by the transmitted light.

Under incident illumination, light-scattering or reflecting chromatophores are brightly visible against the dark background. If we adopt the dark-field epi-illumination system, these cells become more clearly visible, leaving light-absorbing chromatophores practically invisible (Obika, 1976, 1988; Iga, 1978). Using such an optically capable system, Fujii and Miyashita (1979) first tried to apply the photoelectric method for assessing the motile responses of an *Oryzias* leucophore, and they were able to record the leucophore responses without being affected by the motile activities of neighboring melanophores and xanthophores.

However, since the scattering of light from leucophores was faint, highly sensitive photoelectric transducers had to be employed. Thus, Fujii and Miyashita (1979) used a photomultiplier tube for this purpose. In the present study, we used high performance silicon photodiodes coupled with appropri-

ately designed electronic circuits. Taking advantage of the smaller size of the photodiodes, furthermore, we were able to install four of them inside a small housing on the photographic column of the microscope. As another benefit, there was no need for a high voltage supply which is indispensable for the photomultiplier and thus the system could be operated more easily.

When leucosomes are aggregated into the perikaryon of a leucophore, the light scattering there becomes unexpectedly high. As a matter of fact, it was frequently noted that the net amount of light scattering from the entire cell did not change much during the motile response. Namely, light scattering from the entire cell does not necessarily increase with the progress of leucosome-dispersing responses. In order to overcome this problem, Fujii and Miyashita (1979) absorbed the light-rays scattered from the central part of the cell. Ignoring changes in the light scattering from the cell body, they recorded the response by measuring only the changes in light scattering from the dendritic zone of the cell. Actually, the movement of leucosomes away from the center of the cell results in a considerable decrease in the light scattering. In the present study, we have accounted for the changes in light scattering from the cell body, and have succeeded in recording the response more plausibly and precisely.

At this point, we should express our opinion as to which of the three treatments described is preferable for practical purposes. In the second treatment, we do not need the intricate rooter circuit. Usually, however, the linear relationship of the leucosome-dispersing response may result in a problem, observable as the concave curve C in Fig. 6. The electronic processing for the third treatment necessitates both divider and rooter circuits, and inevitably, the operation of the apparatus becomes more complicated. In our investigations, however, this third method has brought about better performance than the second treatment. If one can appropriately set the apparatus to bring the point of inflection of the function $\sqrt{V_p/V_c}$ about the middle of the recording range, the motile response can be recorded along a practically linear function (Fig. 6). Since the relation of the final function to the cellular response is primarily based on the ratio of the two primary functions,

the final values to be recorded on the chart are within a rather limited region. Thus, the net amplification factors ($A_1$, $A_2$) do not require large changes, irrespective of the size of the cell or the amount of light-scattering materials within. In addition, the position of pen on the recorder can be adjusted by applying relatively small values of cancellation voltages, normally within the range of values that can be applied by recorder itself. As examined in the final part of the Results section, the linearity of the response can be improved sufficiently when the input biases to the divider circuit are adequately applied.

The first method is based on a more simple mathematical treatment, namely, that the relation derived from the two primary linear relations is again linear. The electronic part of the system is also simpler, and can be made without difficulty. Depending on the size of the cell or on the amount of light-scattering materials within it, the net amplification factors ($A_1$, $A_2$) should be appropriately adjusted, and the range for adjusting the position of the pen on the chart must be larger than in the other treatments. However, the simplicity of the electronic portions with the ease of handling would be favorable features for novices. For most researchers, therefore, the first method may be recommended.

Employing the first treatment, we have studied the physiology of the motile responses of leucophores and iridophores of the dendritic type. The fact that the present method reflects well the centrifugal or centripetal motion of chromatosomes indicates the appropriateness of the method for studies of motile mechanisms of cell movement. By employing this apparatus, we have found that endothelins disperse leucosomes in leucophores of the medaka (Fujita and Fujii, 1997), and we are now able to investigate the motile mechanisms of leucophores. In any case, reliable results obtained in this manner would surely provide useful information for a deeper understanding of the physiology of pigmentary effector cells in general.

Ödman et al. (1992) recently recorded the response of a melanophore of the cuckoo wrasse Labrus ossifagus by measuring the transmission of light through the center of the cell or through a small circular area within the dendritic zone of the cell. When the measurement was done at the center, dispersion of melanosomes induced an increase in the light transmittance, whereas their aggregation caused a decrease in it. By contrast, when they measured the response within the dendritic part, transmission of light decreased during the dispersal of melanosomes, while it increased when they aggregated into the perikaryon. However, their recording was not of an integrative nature.

When the motile activities of light-absorbing chromatophores are assessed, the conventional transmission photometric method, in which the overall light transmittance through the area occupied by one or more chromatophores is measured, has been successful in many cases (Fujii, 1959; Oshima and Fujii, 1984). In some instances, however, most pigmentary organelles in a chromatophore migrate towards dendritic parts evacuating the cell body almost completely. The increase in the light transmittance through the center of the cell naturally contributes to some extent to the light transmittance through the skin as a whole, and such a phenomenon may be called the "superdispersion". In a photograph presented by Kinosita (1963; Fig. 6), for example, melanophores of Oryzias are seen in such a state. Such a phenomenon seems to occur more remarkably in cells where chromatosomes are rather sparsely distributed. In any case, the net changes of light transmission through the cell are rather limited. By integrating the changes in light transmittance through the central and the peripheral regions, the responses of a single light-absorbing chromatophore can be more plausibly assessed.

In recent years, cells in culture have been frequently employed for analyses of cellular as well as biomolecular mechanisms, including cultured chromatophores from poikilothermic vertebrates. In these cells, application of the conventional method of measuring the light transmitted through the single pathway meets with considerable difficulty since superdispersion of chromatosomes almost always takes place. Working on a urodele species (Ambystoma maculatum), for example, Novales and Novales (1966) went to great effort to measure changes in the area or the perimeter of cultured melanophores to detect the action of melanophore-stimulating hormone. The present method may appropriately be applied to such chromatophores of these animals.

Other studies have employed a video-image analyzing system for assessing the state of chromatophore. For example, we have made good use of such a system to record the responses of tilapia melanophores in culture (Fujii et al., 1991a). However, even such a modern electronic planimetric method often suffers from the same problem since the method is primarily based on the discrimination of the brightness of pixels that constitute the image of the colored cells. In such cases, the photoelectric method reported herein for measuring the transmission of light through different parts of a cell should also be successful.

This method has proven to be very useful, especially when sensitive detection of the effects of some agents on chromatophores is required. We have made good use of this technique for investigating the physiological involvement of novel peptidergic principles, endothelins, on leucophores and on motile iridophores, and detailed results will be presented in a separate paper (Fujita and Fujii, 1997).

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