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ARTICLE

Quantitative Comparison of Anti-Fading Mounting Media for Confocal Laser Scanning Microscopy

M. Ono, T. Murakami, A. Kudo, M. Isshiki, H. Sawada, and A. Segawa

Department of Anatomy, Yokohama City University School of Medicine, Yokohama, Japan (MO,HS); Department of Anatomy, Gunma University School of Medicine, Maebashi, Japan (TM); Department of Anatomy, Kyorin University School of Medicine, Tokyo, Japan (AK); The Fourth Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan (MI); and Department of Anatomy, School of Medicine, Kitasato University, Sagamihara, Japan (AS)

SUMMARY Fading is one of the major obstacles to reliable observation in fluorescence microscopy. Using a confocal laser scanning microscope (CLSM) coupled to a computer, we quantitatively measured fading of fluorescence to formulate an equation, evaluated the anti-fading ability of several anti-fading media, and restored the faded images to the original level according to this equation. NIH 3T3 cells were stained with fluorescein isothiocyanate (FITC)–phalloidin, mounted with several commercial and homemade anti-fade media, and observed with CLSM under repeated illumination. With any mounting medium, attenuation of fluorescence intensity at a certain pixel occurred stepwise and the decrease was proportional to the intensity of the previous scan. From these results, we formulated an equation that has three coefficients: anti-fading factor (*A*), indicating the ability to retard fading; fluorescent intensity at the first scan (EM_1); and background fluorescence (*B*). The fluorescent intensity at a certain point following *n*th scan is given as $EM_n = EM_1 \bullet A^{(n-1)}$. This equation was available for restoring faded images to their original states, even after the image had faded to only 60% of its original intensity.

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KEY WORDS
anti-fading media
anti-fading factor
confocal laser scanning microscopy

IN FLUORESCENCE OBSERVATION by conventional fluorescence microscopy and confocal laser scanning microscopy (CLSM), the retardation of fluorescence fading, the high initial intensity of images, and low background noise are important factors for obtaining clear and accurate images. Fluorescein isothiocyanate (FITC) is the most widely used fluorochrome in fluorescence microscopy. However, the fluorescence of FITC is rapidly lost when it is exposed to excitation light, and FITC-stained preparations mounted in conventional buffered glycerol show prompt bleaching under illumination, especially under optimal conditions for observation: under illumination at the wavelength of maximal absorbance of the fluorochrome and with objective lenses of a high numerical aperture (Johnson et al. 1981; Benson et al. 1985). The photochemical process underlying the fluorescence decay of FITC has not yet been fully explained, although theories suggesting the involvement of oxygen, triplet states, and protein denaturation have been proposed (Hirschfeld 1979; Johnson et al. 1982). Preventing the fading of fluorescence intensity caused by the excitation light is very important for obtaining stable and accurate images. Reagents that also defer drying and fading during storage and CLSM observation are widely used for this purpose.

Several types of mounting media are available (Gill, 1979; Johnson et al. 1981,1982; Giloh and Sedat 1982; Harris 1986; Krenik et al. 1989; Longin et al. 1993), such as *p*-phenylenediamine (PPD) (Johnson et al. 1981,1982; Platt and Michael 1983), *N*-propyl gallate (NPG) (Giloh and Sedat 1982), and 1-4-diazabicyclo[2,2,2]-octane (DABCO) (Johnson et al. 1982; Langanger et al. 1983). Ready-to-use anti-fading kits are also commercially available.

In this study, we examined the fluorescence fading phenomenon quantitatively, obtained an equation to

Correspondence to: Michio Ono, Dept. of Anatomy, Yokohama City U. School of Medicine, 3-9, Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. E-mail: mono@med.yokohama-cu.ac.jp

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express the phenomenon, compared the ability of various anti-fading media, and tried to restore already faded images to their original state with a computer.

Materials and Methods

Cell Preparation

NIH 3T3 cells were used for this study. The cells were cultured on coverslips (22 mm^2) in RPMI-1640 medium containing 10% newborn calf serum in a humidified atmosphere of 5% CO_2 and 95% air at 37C. Nonadherent cells were removed by rinsing the coverslips with PBS. The cells on coverslips were fixed and permeabilized with 4% paraformaldehyde and 0.05% Triton X-100 in PBS (pH 7.4) for 10 min at room temperature (RT), washed with PBS, and then stained with 0.1 mg/ml FITC–phalloidin (Sigma Chemical; St Louis, MO) and stored overnight at 4C. The coverslips were washed three times with PBS and mounted with different media, as described below.

Mounting Media

We used 50% glycerol in 20 mM phosphate buffer (pH 8.5) (GB) as a glycerol-based standard medium. For experiments with homemade anti-fading reagents, 0.1% *p*-phenylenediamine (PPD) (Wako Pure Chemical Industries; Tokyo, Japan) (Johnson et al. 1981,1982) or 2.5% 1,4-diazabicyclo-[2,2,2]-octane (DABCO) (Wako Pure Chemical Industries) (Johnson et al. 1982; Langanger 1983) was added to the GB. We also used polyvinyl alcohol-based anti-fading mounting medium consisting of 2.5% DABCO, 10% polyvinyl alcohol (PVA) (Sigma; Type II), 5% glycerol, and 25 mM Tris buffer, pH 8.7 (Valnes and Brandtzaeg 1985). All homemade anti-fading reagents were freshly prepared before use.

As commercial anti-fading media, we used SlowFade Light Antifade Kit (Molecular Probes; Eugene, OR), PermaFluor (Lipshaw/Immunon; Pittsburgh, PA), FluoroGuard Antifade Reagent (Bio-Rad Laboratories; Hercules, CA), and ProLong Antifade Kit (Molecular Probes). The commercial media were used within 1 month because those were newly opened. All specimens were prepared in the same way before observation. The plastic tapes that opened the rectangular hole were used as spacers for cultured cells on coverslips. The spacer was placed on a glass slide. Then the hole was filled with each mounting medium. The coverslips were put upside down on the spacer, then, were fixed with glue. The mounted specimens were incubated for 1 hr in the dark before use.

Confocal Laser Scanning Microscopy

We used a confocal laser scanning microscope (LSM GB-200; Olympus, Tokyo, Japan) coupled with an IEEE-488 interface to an IBM PS/V 486-66M computer (IBM). The excitation source was an argon ion laser with a 20 mW output power at 488-nm line and 1%, 3%, and 10% transmittance normal-density (ND) filters. The fluorescence emission was split by a dichroic mirror DM488 and was measured by a detector placed behind a BP530-nm bandpass filter.

Fluorescence emission was recorded through a ×60 SPlan-Apo oil-immersion objective with a high numerical aperture (1.4). Each specimen was scanned 50 times and the whole scanning field was recorded for each scan. The area and the rate of laser scanning were 1024 × 768 pixels/40 sec (0.257 μm/pixel and 51 μsec/pixel). The conditions for high-voltage, gain, and offset were −800 kV, 2.0, and 2, respectively, and the same conditions were used throughout the study. The images were stored on the coupled personal computer. All measurements were performed at RT.

Data Analysis and Image Processing

We analyzed fluorescence intensity by fading in pixel units, using a Power Macintosh 7300/166 (Apple Computer; Cupertino, CA). For the analysis, we developed software (AF Analyzer) to obtain fluorescence intensity of the same coordinate pixels from every excitation. For each pixel, the mean value of initial fluorescence intensity was compared with the intensities after fading by using the AF Analyzer. These data were analyzed using the pixel data from which saturated intensity and background intensity were subtracted. The regression lines were calculated and drawn with MacCurveFit (Kevin Raner Software, Victoria, Australia; http://www.home.aone.net.au/krs/).

We performed image processing for correction of fading with FluoroFixer software (http://bioimage.med.yokohama-cu.ac.jp/confocal/fluorofixer), which we developed to enable fluorescence reconstitution calculated by the use of a fading equation.

Results

The Nature of the Decrease in Fluorescence Intensity

To ascertain the ability of anti-fading reagents to reduce the decrease in normalized fluorescence intensity, we compared the time course of bleaching of FITC–phalloidin-stained NIH 3T3 cells in mounting media

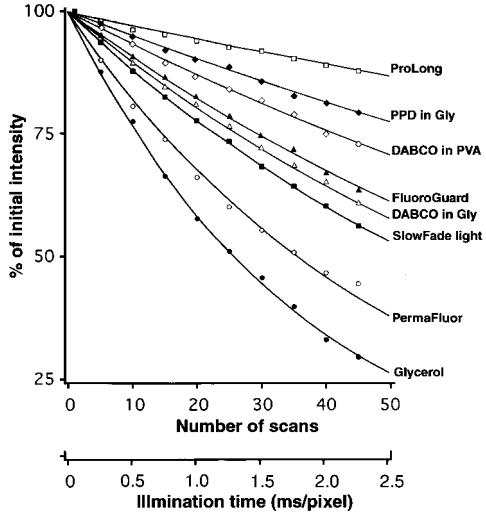

Figure 1 Nonlinear least-squares analysis on fading of fluorescence intensity with several commercial and homemade anti-fading media. Normalized intensities of each image were used as data. All the measurements were done with a 3% ND filter.

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with or without anti-fading reagents under a CLSM (Figure 1). With all the mounting media tested, the fluorescence decreased as a function of the number of scans, although the decreasing speeds and the initial fluorescence intensity were significantly different among the media. It was not possible, however, to compare quantitatively the ability of different mounting media to prevent a decrease in fluorescence with this method, because the slope of the graph changed as the number of scans increased even for a single mounting medium.

In the method used in Figure 1, there is also the possibility that the speed of fading is different among spots with different initial fluorescence intensities. We decided to take advantage of the fact that in CLSM the intensity of each spot can be recorded digitally, and the decrease in fluorescence intensity in each pixel unit can be traced. To compare the change in fluorescence intensity between spots with different initial intensities, we measured the change in fluorescence intensities of all the spots and grouped them according to the initial intensities. Figure 2 shows the data for eight different initial intensities of a buffered glycerol (GB)-mounted specimen. The stronger the initial fluorescence intensity, the more rapidly the intensity decreases in response to laser illumination. We again recognized that the amount of fading decreases as the number of scans increases.

An Equation to Indicate Fluorescence Fading

In the CLSM, because the specimens are illuminated intermittently, the intensity of fluorescence decreases in a stepwise manner between each scan, and the quantity of fading can be measured as the difference in the intensity between scans. We measured the difference in intensities between adjacent spots and plotted them as a function of the fluorescence intensities in the previous scan (Figure 3). This value is shown to be almost in proportion to the fluorescence intensity in the previous scan for each mounting medium, and the lines fit very well when we employed a linear least-squares fit. We determined that the relation of the fluorescence intensity and the quantity of fading by illumination is given by the following equation:

$$\Delta EM = k \bullet EM \qquad (1)$$

where ΔEM is the decrease in fluorescence intensity, k is the inclination of regression lines in Figure 3 and thus a coefficient that indicates the extent of fading, and EM is the fluorescence intensity in the previous scan. Equation 1 can be changed to $EM_{n+1} = EM_n (1 - k)$, then into:

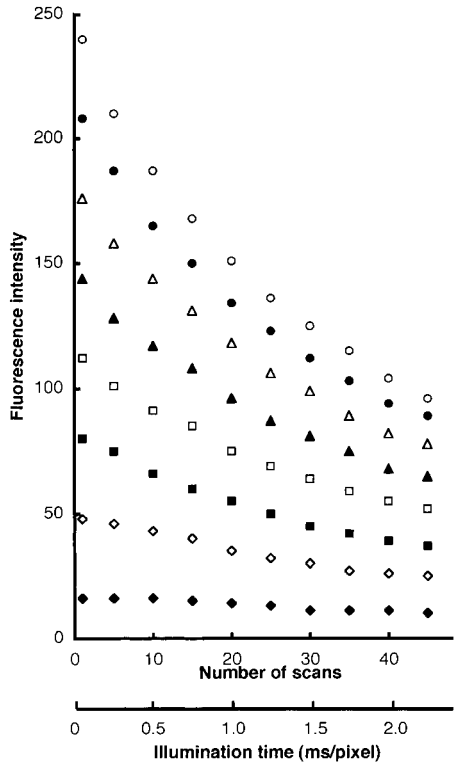

Figure 2 Fading profiles of fluorescence of FITC–phalloidin-stained NIH 3T3 cells mounted in buffered glycerol during CLSM exposure. Eight groups of pixels that showed initial intensities (intensities at the first scan) of 240 (○), 208 (●), 176 (△), 144 (▲), 112 (□), 88 (■), 48 (◇), and 16 (◆) were selected, and then the fluorescence intensities of each spot at the indicated scans were recorded and averaged for each group; 3% ND filter.

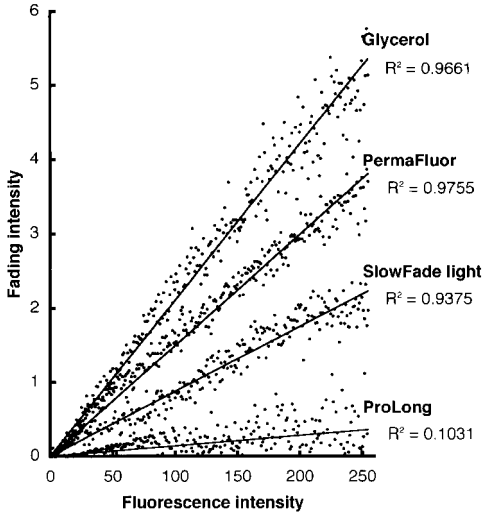

Figure 3 Fluorescence fading of FITC–phalloidin-stained NIH 3T3 cells mounted in various anti-fading media during exposure to CLSM excitation. The decreases in intensity at certain scans were plotted to fluorescence intensities in the previous scans. The data were calculated from the data at each pixel. The lines show a linear least-squares fit (R^2, coefficient of determination). All measurements were done with a 3% ND filter.

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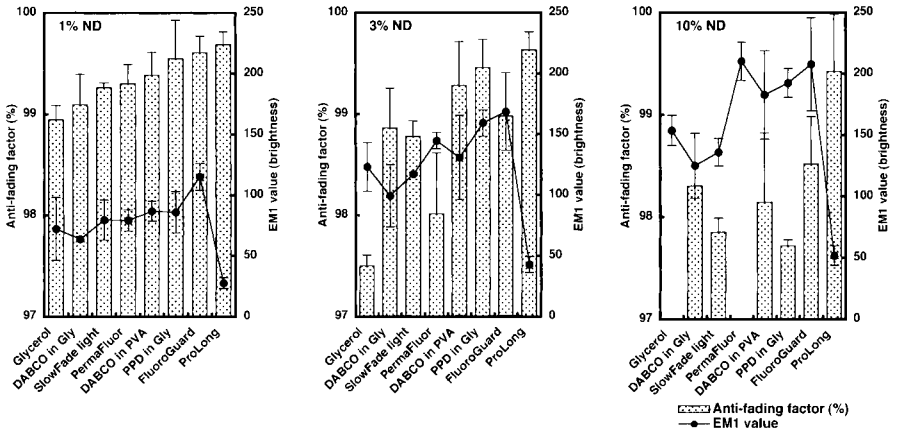

Figure 4 Anti-fading factor (bar) and EM_1 value (line) of the anti-fading function of the mounting reagents tested ($n = 4$). These coefficients were obtained by a nonlinear least-squares analysis. Vertical lines, SD.

$$\frac{EM_{n+1}}{EM_n} = (1-k) = A \qquad (2)$$

where EM_n is the fluorescence intensity of the nth scan. Equation 2 is a geometrical progression in which A is the common ratio. A is a constant between 0 and 1 that is specific for each mounting media.

$$EM_n = EM_1 \bullet A^{(n-1)} \qquad (3)$$

When a background intensity (B) which does not fade is introduced, the equation is changed to:

$$EM_n = (EM_1 - B) \bullet A^{(n-1)} + B \qquad (4)$$

For more general uses, we can use time (t) instead of the number of scans (n). In this case, Equation 1 can be converted to

$$EM_t = EM_o \bullet e^{-kt} \qquad (3')$$

Equation 3′ can be changed to the following by setting $A' = 1/k$. Then the following equation can be deduced from Equation 4 by using (t) instead of (n).

$$EM_t = (EM_o - B) \bullet e^{-\frac{t}{A}} + B \qquad (4')$$

A' is the time spent until fluorescence intensity becomes 1/e, which is specific for each mounting medium. The larger A and A' are, the stronger the ability of a given mounting medium to prevent fading. Therefore, we defined A as the anti-fading factor in the study. Thus, we were able to obtain an equation to evaluate the ability of a medium to retard fading. With this equation, the fluorescence intensity after a given number of scans can be calculated from the original intensities (intensities at the first scan) as described below. We call these equations, either Equation 4 or Equation 4′, the fading equation in this study.

Comparison of Mounting Media by Fading Equation

We compared the ability of anti-fade media using the fading equation. We calculated means of intensities of all pixels in each scanned image and calculated A and EM_1 values for each anti-fading medium by a nonlinear least-squares analysis (Figure 4; Table 1). The B value was adjusted to 0 by selecting an optimal offset value. Among all the anti-fading media tested, ProLong showed the highest A values under both high (99.423 at 10% transmittance ND) and low (99.635 at 1% transmittance ND) excitations, indicating the strongest anti-fading effect, but showed the lowest EM_1 value, which indicates the darkest initial image. FluoroGuard showed the second highest A value (99.607) and a high EM_1 value, indicating that FluoroGuard gives both a strong anti-fading effect and bright initial images. PPD and DABCO also showed good anti-fading effects comparable to some commercially available anti-fading media, but the effects were not as strong as those of ProLong or FluoroGuard. PPD appears to give higher EM_1 values than DABCO. As for the diluents, glycerol and PVA did not show a

Table 1 Anti-fading factor and EM_1 value of the fading equation of the mounting reagents tested ($n = 4$)

Mounting media	1% ND	3% ND	10% ND
Anti-fading factor			
Glycerol	98.94 ± 0.14	97.50 ± 0.11	93.53 ± 0.09
DABCO in Gly	99.09 ± 0.30	98.86 ± 0.39	98.30 ± 0.25
SlowFade light	99.26 ± 0.05	98.78 ± 0.15	97.85 ± 0.14
PermaFluor	99.30 ± 0.19	98.01 ± 0.60	93.73 ± 1.15
DABCO in PVA	99.38 ± 0.23	99.28 ± 0.43	98.14 ± 0.68
PPD in Gly	99.55 ± 0.38	99.46 ± 0.28	97.72 ± 0.06
FluoroGuard	99.61 ± 0.16	98.98 ± 0.17	98.51 ± 0.47
ProLong	99.69 ± 0.13	99.63 ± 0.18	99.42 ± 0.56
EM_1 value (brightness)			
Glycerol	72.2 ± 25.9	122.9 ± 20.1	153.9 ± 12.3
DABCO in Gly	63.8 ± 2.2	99.2 ± 25.6	125.0 ± 26.4
SlowFade light	79.5 ± 16.4	116.9 ± 1.0	136.0 ± 11.3
PermaFluor	79.2 ± 8.7	144.7 ± 6.8	210.2 ± 15.6
DABCO in PVA	86.9 ± 8.2	130.8 ± 34.8	182.7 ± 36.0
PPD in Gly	85.9 ± 17.1	159.2 ± 10.8	192.3 ± 11.8
FluoroGuard	115.0 ± 11.0	168.5 ± 32.0	207.7 ± 38.0
ProLong	47.6 ± 4.3	73.7 ± 6.6	89.5 ± 7.8

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big difference in A values, but PVA containing diluent seemed to be superior in terms of initial intensities (EM_1).

Correction of Fading Image by the Fading Equation

Because the alteration in fluorescence due to fading is provided by the fading equation, the initial illuminated intensities of a fluorescent image can be calculated from the intensities after a given number of scans with the following equation, which is calculated from Equation 4:

$$EM_1 = \frac{EM_n - B}{A^{(n-1)}} + B \qquad (5)$$

We attempted to restore an original image with this equation, using a personal computer, from an image that had faded as a result of prolonged illumination. Figure 5 shows FITC–phalloidin-stained NIH 3T3 cells mounted in buffered glycerol which had been faded by prolonged illumination (Figures 5B–5D) and the images restored to their original luminescence with Equation 4 (Figures 5E–5G). Restoration without significant deterioration of the images that had faded up to 60% of their initial fluorescence intensity was possible with this equation (Figure 5G compare with Figure 5A). The equation not only is available to indicate the abilities of anti-fading media but it can also be available for the correction of faded images.

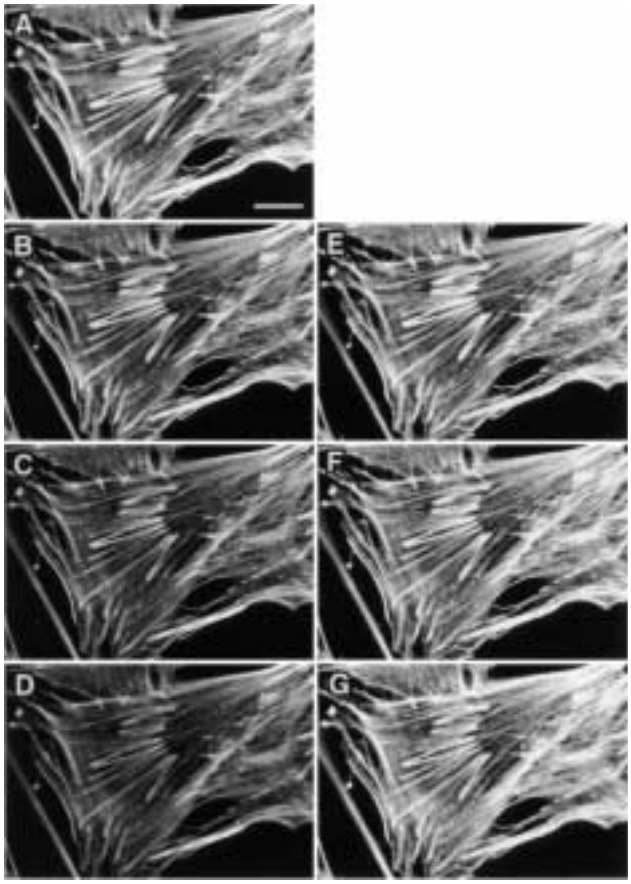

Figure 5 Faded images caused by prolonged excitation and their restoration with the fading equation. The restoration was processed with FluoroFixer. CLSM images of FITC–phalloidin-stained NIH 3T3 cells mounted in buffered glycerol. **A–D** show initial images (**A**), and the images faded to approximately 80% (**B**), 60% (**C**), and 40% (**D**) of the initial intensity. **E**, **F**, and **G** show images restored with the fading equation from faded images **B**, **C**, and **D**, respectively. Bar = 10 μm.

Discussion

Fading Equation

We compared the ability of various commercial and homemade anti-fading media by CLSM. In previous reports, normalized intensities of images obtained by every scan were compared (Johnson et al. 1982; Böck et al. 1985; Krenik et al. 1989; Longin et al. 1993; Florijn et al. 1995), and the time needed for the intensity to decrease by 50% was measured (Longin et al. 1993), or regression curves on logarithmic graphs were drawn and the inclinations obtained by linear least-squares analyses were compared (Giloh and Sedat 1982). In these methods, there is no guarantee that the speed of fading is equal between spots of different initial fluorescence intensities, and it was not verified whether these methods gave a reliable comparison of different mounting media or a quantitative analysis of fading. Tsien and Waggoner (1995) described photophysics and photochemistry for the laser-scanned digital image. In the present study, taking advantage of the digital nature of CLSM images, we examined the nature of fluorescence fading during illumination at each pixel unit and obtained Equation 3, whose exponential function fits very well with our experimental observations.

Anti-Fading Factor

The decrease in fluorescence intensity after each scan was shown to be proportional to the intensity before the scan at the pixel unit, regardless of the fluorescence intensities (Equation 1). Consequently, the decrease of intensity by excitation was shown as an exponential decay function (Equations 2–4). This fact can be explained as follows. In CLSM, fluorochromes under incident light are excited and emit fluorescence. A certain proportion (F in Equation 1 or $1\text{-}A$) of the excited fluorochromes are oxidized and will no longer emit fluorescence (Hirschfeld 1979; Johnson et al. 1982). This is the phenomenon of photobleaching, which is shown in Equation 4. The characteristics of fading media can be expressed with three coefficients: A (a constant that is specific for each mounting medium), EM_1 (initial intensities), and B (background in-

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tensities). The coefficient A is the general form of the geometrical progression. Its value is between 0 and 1, and the larger the A value, the stronger the mounting medium's ability to prevent retardation of fluorescence intensity. Therefore, we defined this factor as the "anti-fading factor" of anti-fading media. Coefficient EM_1 also varies among different mounting media.

To prevent retardation of fluorescence intensity, three methods can be used. The first is to remove oxygen from the mounting medium. The second is to increase the viscosity of the mounting medium and retard the diffusion of oxygen, such as with the use of glycerol or PVA as a mounting medium. The third is the use of a reagent that quenches the excitation and consequently reduces the amount of oxidized fluorochromes. Azide, iodide ions, and DABCO are known to have this quenching ability (Johnson et al. 1982). In this method, however, although the fluorescence does not bleach quickly, the intensity of fluorescence should not be high. Judging from the result of our experiment, ProLong appears to use this effect.

In photochemistry, the Stern–Volmer equation is used to express the process of quenching (Barltrop and Coyle 1978). Reagents' ability to quench can be quantitated by the coefficients of the equation. In practical immunochemistry, however, the above three mechanisms are used in combination to prevent fluorescence attenuation. In this situation, commonly used reaction kinetics such as the Stern–Volmer equation cannot be applied because of the high viscosity of mounting media. Moreover, in the case of the commercial anti-fading media in which concentrations of constituents cannot be changed arbitrarily, the coefficients of the Stern–Volmer equation cannot be determined. For that reason, we had to resort to our equation to assess the ability of anti-fading media.

Comparison of Anti-Fading Media and Their Practical Use in CLSM

Various mounting media were compared with their anti-fading factor (A) and initial intensity of fluorescence (EM_1). Among commercial and homemade anti-fading media examined, ProLong showed the highest A value. Its A value remained high even under strong excitation. ProLong, however, has a low EM_1 value. On the other hand, FluoroGuard showed the second highest A value and a relatively high EM_1 value. However, the A value of FluoroGuard decreases when excitation is strong. In general, in high EM_1 (bright) mounting media, more fluorochromes are excited at the same illumination intensity than in low EM_1 mounting media. Accordingly, the decrease in fluorescence intensity cannot be prevented well in high EM_1 mounting media. Because with CLSM the brightness and contrast of images can be controlled arbitrarily by adjusting the sensitivity of photomultipliers, gain, and offset, we can use anti-fading media with high retardation ability even though they may have low EM_1 values. In this context, Longin et al. (1993) reported that both high initial intensity and high retardation ability are obtained by mixing a bright mounting medium with a mounting medium of high retardation ability. However, we have not tried mixing different media yet. Recently, new fluorescent Alexa dyes became available. They are remarkably brighter and more photostable than conventional fluorescence dyes (Landon 1997). With these fluorochromes, the A value is more important than the EM_1 value, and anti-fade media with high A values, even if they have low EM_1 values, as with ProLong, are more advantageous than other dyes (Panchuk–Voloshina et al. 1999).

Application of the Fading Equation

The application of our fading equation enables us to restore faded images. We can calculate the initial intensities of each pixel unit with our fading equation backward from the faded images. As shown in Figure 5, recovering the original state from an image faded to 60% of its original fluorescence was possible. This application is particularly important for specimens in which an anti-fading reagent cannot be used. For example, in fluorescence observation of living cells, it is difficult to use an anti-fading agent, and therefore it is difficult to distinguish physiological changes in fluorescence intensity from changes caused by fading. Another application is to reconstruct quantitative 3-dimensional images. In 3D observation of CLSM, the specimens fade during scans on various optical planes by repeated excitations. Each plane is faded during the illumination to other planes, which should be corrected (Centonze and Pawley 1995). We are currently engaged in contriving a method for reconstituting quantitative 3D images.

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