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Antifading Embedding Media in Confocal Immunoflourescence Microscopy

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Fading or bleaching of fluorescence intensity during continuous illumination of stained objects is a serious problem in fluorescence microscopy. Fluorescence intensity as well as bleaching characteristics of dyes are dependent primarily upon physical parameters such as molecular constants (absorption rate and quantum efficiency), excitation energy and brightness (causes photon saturation), and environmental parameters (pH, ions, binding to proteins, etc.) that can strongly influence the properties of fluorochrome molecules. We have studied the effect of various antifading reagents on the behavior of the common dyes fluorescein isothiocyanate (FITC) and phycoerythrin (PE) using immunofluorescent-stained living cells in suspension or membrane-permeabilized dried cells as test systems. As expected, fading cannot be completely eliminated but may be reduced to varying degrees. In our hands, the most efficient antifading reagent for FITC is *n*-propyl gallate (NPG) dissolved in glycerol. No additive was found to retard fading, but complete dehydration of the cell suspension reduces this effect.

KEY WORDS: Immunofluorescence; bleaching; laser scanning microscopy.

INTRODUCTION

Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) are common dyes used in immunofluorescence microscopy and also in flow cytometry for single and double-labeling, due to their high absorption rate, quantum efficiency, and ability to bind to proteins without compromising their function. One disadvantage is the sensitivity of these dyes to illumination and subsequent fading or bleaching. This phenomenon is particularly important in confocal microscopy, where detection of low fluorescence intensities requiring a low scanning speed and/or an additive effect of repeated scans is essential to obtain satisfying signal/noise ratios and, therefore, a proper image.

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A comprehensive theory explaining the phenomenon of fluorescence bleaching is still lacking, although it seems not to be the result of a single reaction. Excited dye molecules can follow different pathways, including irreversible bleaching (photodestruction, probably caused by oxidation of the molecule), triplet excitation, or transition through some kind of "semioxidized" state with the potential of a reverse reaction.

For the DNA stains Hoechst 33258 and propidium iodide, Van den Engh's data [14] concerning photon saturation and bleaching in flow cytometry are in good agreement with his signle photon model theory. Lindquist [7] showed that free fluorescein in an aqueous solution is converted into a triplet state, followed by formation of nonfluorescent radicals possessing the potential for a reverse reaction. This model explains the recovery phenomenon, or the partial restoration of fluorescence in bleached samples after a dark interval [11,12].

A variety of possible remedies has been recommended to overcome or retard bleaching, among them

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the use of antibleaching reagents as embedding media. In general, embedding media play an important role in fluorescence microscopy: They should be transparent and nonfluorescent, provide physiological conditions (conductive to maintaining the structure of the cells under study), immobilize cells, and avoid fluorescence bleaching. In confocal microscopy, these requirements are even more important because of the sensitive fluorescence detection system, the high resolution in the X/Y direction, and the possible reconstruction of the Z-plane, which requires great accuracy in specimen preparation [1]. Many embedding media and additives have been proposed as antibleaching reagents for cell suspension and tissues or cells growing on surfaces [3–6,8–10,13]. Previous work in our laboratory compared antibleaching reagents with a laser microscope for FITC [2]. In the present study, we repeated these measurements with a confocal microscope and also assessed these antifading reagents for PE staining.

MATERIALS AND METHODS

Microscopic Equipment

A laser scanning fluorescence microscope (LSM10, Zeiss, Oberkochen, Germany) was used for all bleaching experiments. The LSM 10 is equipped with a 63/1.4 oil-immersion objective and a 5-mW argon laser (488 nm) for the scanning mode (scanning area, 100 × 50 μm); the scanning time was 2 s. The resulting fluorescence images from the first and the fifth scan were stored in a computer and all pictures were photographs taken from a video monitor with identical exposure times.

For visual fluorescence reading and quantification of fluorescence intensities, a conventional mercury vapor bulb (HBO50) was used together with a photomultiplier tube (PMT; Hamamatsu R928, Hammatsu City, Japan; operating voltage, 600 V; combined with a preamplifier Module H, Seitner Messtechnik, Seefeld, Germany). An aperture was set in front of the PMT, permitting light to pass only from the center of the viewing field of the microscope. The PMT signal (passing an electronic low-pass filter to reduce noise) was detected by a x/t recorder.

Cell Preparation

Human peripheral blood lymphocytes (EDTA-blood, erythrocytes removed by lysis) were stained with either monoclonal FITC-labeled anti-CD3 antibodies or PE-labeled anti-CD4 antibodies (both from SeraLab, Sussex, GB), 5 μl/100μl whole blood.

After staining and washing with phosphate-buffered saline (PBS; pH 7.2), the cells were embedded in various media on the microscope slide, covered with a coverslip, the edges of which were seated with clear nail polish.

RESULTS

FITC-Labeled Cells

Figure 1 a fluorescent CD3+ cell suspended in PBS (left, first scan; right, fifth scan). Almost no fluorescence could be detected after a few scans. Figure 2 shows the fluorescence of a similar cell, embedded in *n*-prophyl gallate (NPG), dissolved in 40 mg/ml glycerol (left, first scan; right, fifth scan). Bleaching is clearly reduced compared to Fig. 1.

PE-Labeled Cells

In Fig. 3 the fluorescence of a CD4+ cell (left, first scan) suspended in PBS is completely bleached after the fifth scan (right). To remove water from the PE-stained cells (as water is suspected to exacerbate bleaching), cells were first dried on a microscope slide, fixed with ethanol, dried again, and embedded in liquid paraffin, then covered with a coverslip, and the edges were sealed. As evident in Fig. 4 fluorescence is well preserved under these conditions between the first (left) and the fifth (right) scan.

Quantification of Bleaching

Figure 5 shows fluorescence intensities (FI; relative units) of single stained cells (top, FITC-labeled cells; bottom, PE-stained cells) prior to the first laser scan and after the fifth scan. Again, the protective effect of NPG (for FITC) and drying and embedding in paraffin (for PE) is clearly demonstrated.

DISCUSSION AND CONCLUSION

FITC fading can be reduced by additives to the embedding media, which is important for the preservation of the three-dimensional structure of cells. Of the many additives tested in our laboratory (data not shown), *n*-propyl gallate best reduced bleaching. None of the additives mentioned in the literature for FITC can retard fading of PE-labeled cells (data not shown). In the latter instance, fading can be reduced only by the complete

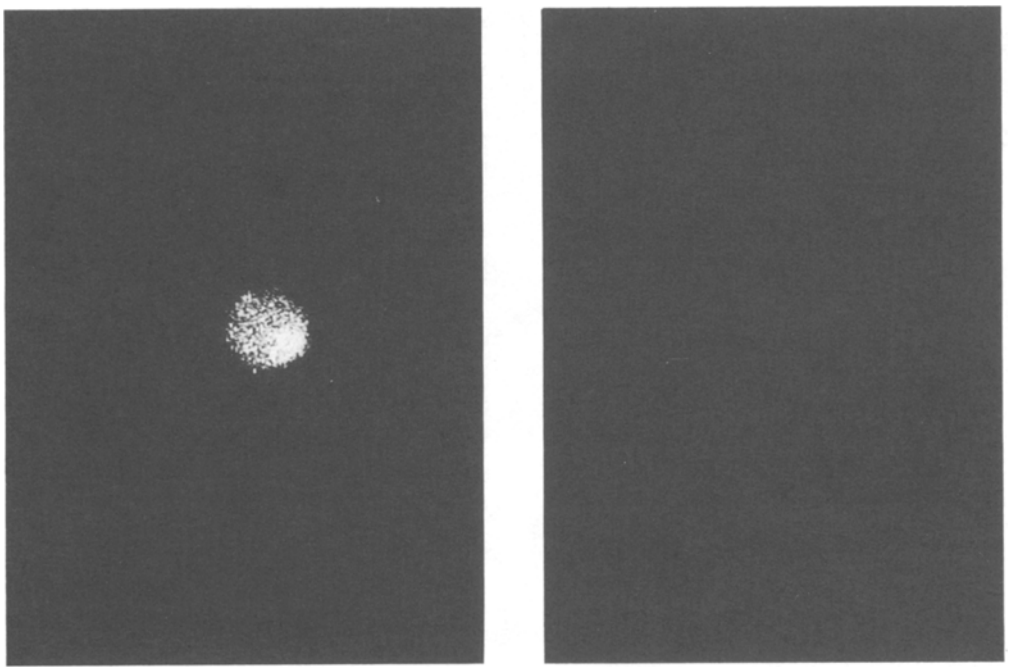

Fig. 1. FITC-stained cell (CD3+) suspended in PBS. Left, fluorescence from the first laser scan; right, from the fifth scan.

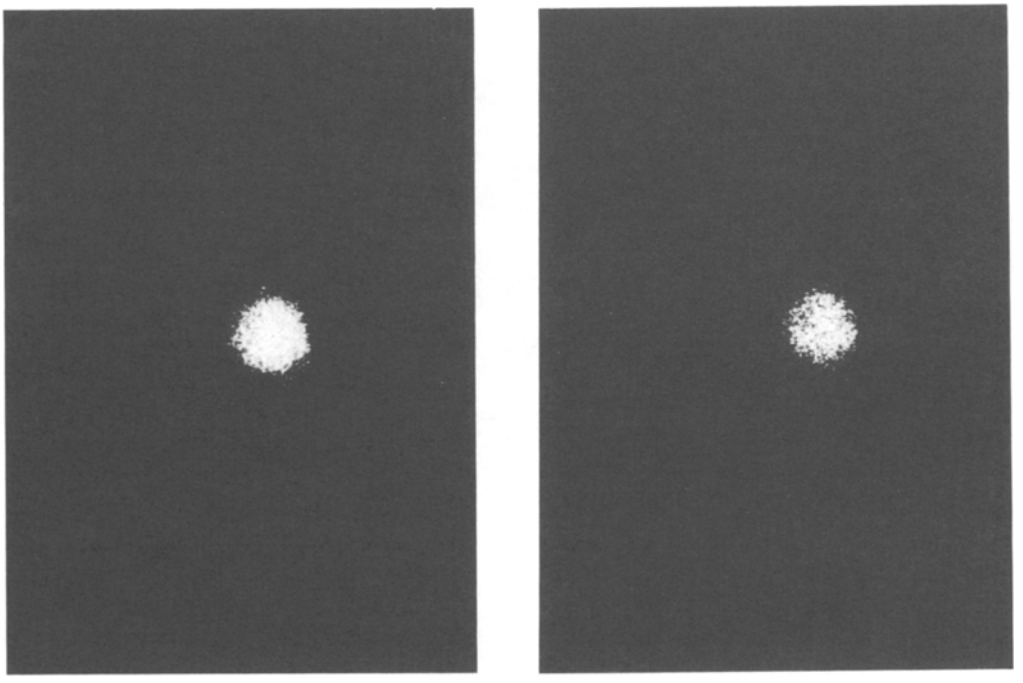

Fig. 2. FITC-stained cell (CD3+) embedded in the antibleaching reagent NPG. Left, fluorescence from the first laser scan; right, from the fifth scan.

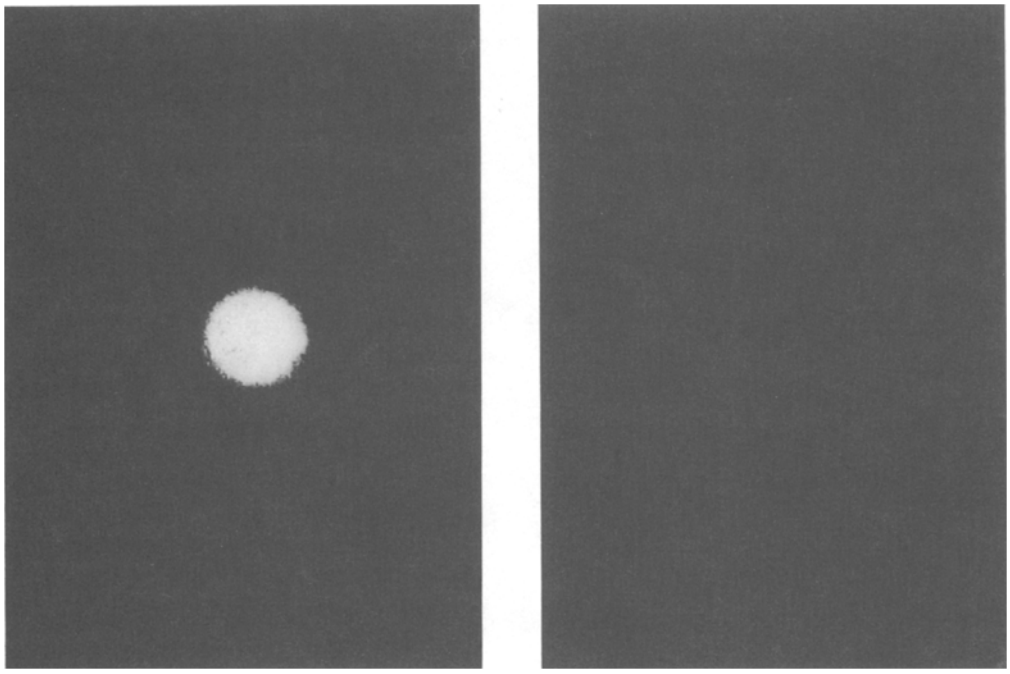

Fig. 3. PE-stained cell (CD4+) suspended in PBS. Left, fluorescence from the first laser scan; right, from the fifth scan.

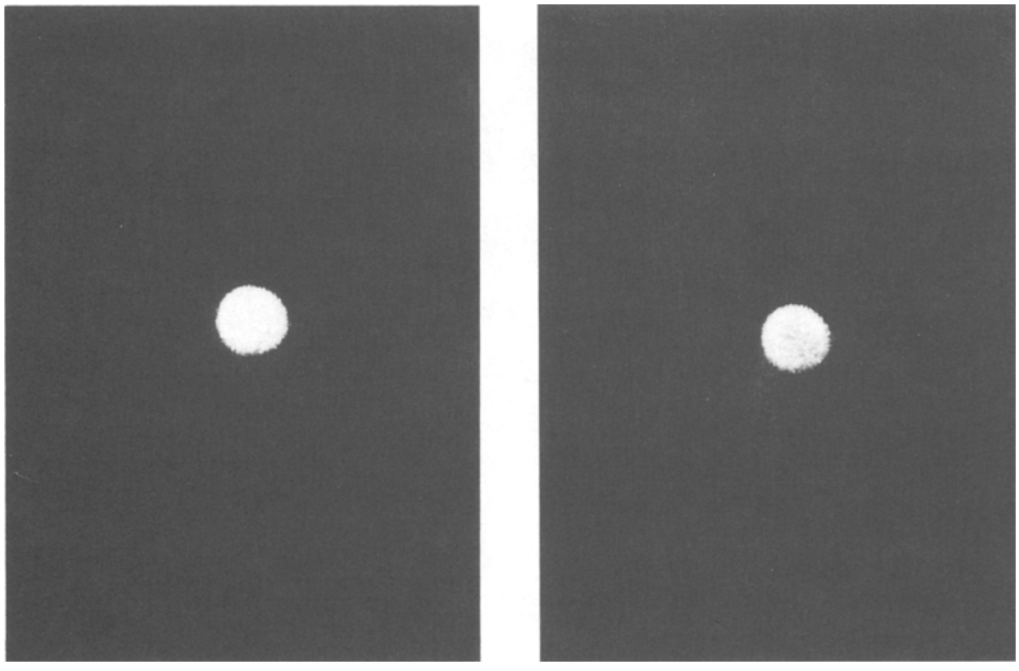

Fig. 4. PE-stained cell (CD4+) dried and embedded in liquid paraffin. Left, fluorescence from the first scan; right, from the fifth scan.

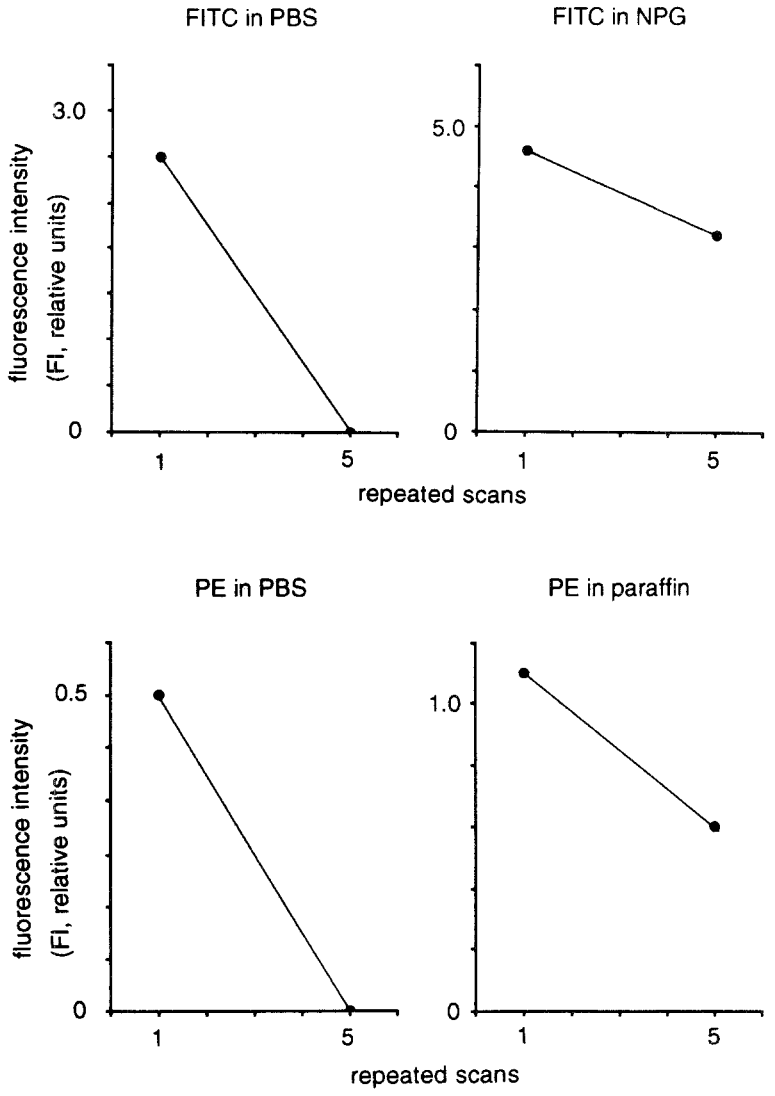

Fig. 5. Quantification of FITC (top) and PE (bottom) bleaching. Fluorescence intensities of single cells prior to the first and after the fifth scans. Left, cells in PBS; right, cells in antifading embedding medium.

removal of water, which is guaranteed by cell drying and embedding in liquid paraffin. Similar good results can be obtained with oily media. The described dry emedding also reduces FITC fading. The main disadvantage of cell drying is, of course, a loss of three-dimensional cell structure.

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