

BRIEF REPORT

Simple Method for Reduction of Autofluorescence in Fluorescence Microscopy

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SUMMARY Autofluorescence of aldehyde-fixed neural tissue often complicates the use of fluorescence microscopy. Background fluorescence can be notably reduced or eliminated by irradiation with light before treatment with fluorescence probes, resulting in a higher contrast without adversely affecting the staining probabilities. (*J Histochem Cytochem* 50:437–439, 2002)

KEY WORDS
autofluorescence
photobleaching
immunofluorescence

THERE ARE many causes for autofluorescence of tissue. Some exist only in mammalian tissue, such as the fluorescent pigment lipofuscin, which accumulates with age in the cytoplasm of cells. Others are specific to plants or are independent of the species and find their origin in the embedding material. These causes of autofluorescence have one thing in common: They complicate the use of fluorescence microscopy. In the literature, many techniques are described for reducing autofluorescence: CuSO_4 in ammonium acetate buffer or Sudan Black B in 70% ethanol (Schnell et al. 1999), NaBH_4 (Clancy and Cauller 1998), Pontamine Sky Blue (Cowen et al. 1985), or mathematical models that attempt to subtract the background fluorescence because of the broader autofluorescent excitation spectra compared to the spectra of the fluorescent label (Steinkamp and Stewart 1986; Van de Lest et al. 1995).

All these techniques have their disadvantages. Treatment with chemicals also reduces the intensity of immunofluorescent labeling, which demands a compromise between autofluorescence reduction and antigen visualization. The mathematical models, such as the differential fluorescence correction, are difficult and carry the risk of false results.

We have developed a simple method that reduces most kinds of autofluorescence in mammalian tissue without adversely affecting the probability of labeling the tissue with fluorescent markers. We found that irradiation with light reduces all autofluorescence by

photobleaching in tissue sections of both brain and liver.

Human brain tumor tissue from different patients and murine liver tissue were fixed in paraformaldehyde and embedded in paraffin. From these, thin sections (5 μm) were cut, mounted on glass slides, and centered in a box with up to four fluorescent tubes: one conventional neon tube (18 W), one UV tube (20 W), and two tubes from Dennerle, Germany (Trocal 5085 and Trocal-Kombi 8085, 18 W each), which have higher emission peaks at 488 nm and 633 nm, respectively. By choice of the length of the tubes the maximal amount of slides which could be irradiated simultaneously can be varied (in this case 18). For maximal yield of light the box was lined with aluminum foil. The sections were irradiated for 12–48 hr with each tube, depending on the thickness and intensity of the autofluorescence. Some heat-sensible antigens may require cooling while being irradiated, especially when more than two tubes are used simultaneously. After irradiation the paraffin from the thin sections was removed by two 10-min washes in xylene and sections were mounted in Entellan (Merck, Germany). For comparison, neighboring sections of each treated tissue section were deparaffinized and mounted without irradiation. To test the influence of the treatment on the ability of fluorescence markers to label cell structures, samples with and without irradiation were labeled either with ethidium bromide and acridine orange or with indirect immunofluorescence. For the latter, primary antibodies against glial fibrillary acidic protein (anti-GFAP, Z0334; DAKO, Glostrup, Denmark; prediluted) (20-hr incubation at room temperature) were used. The secondary antibody was

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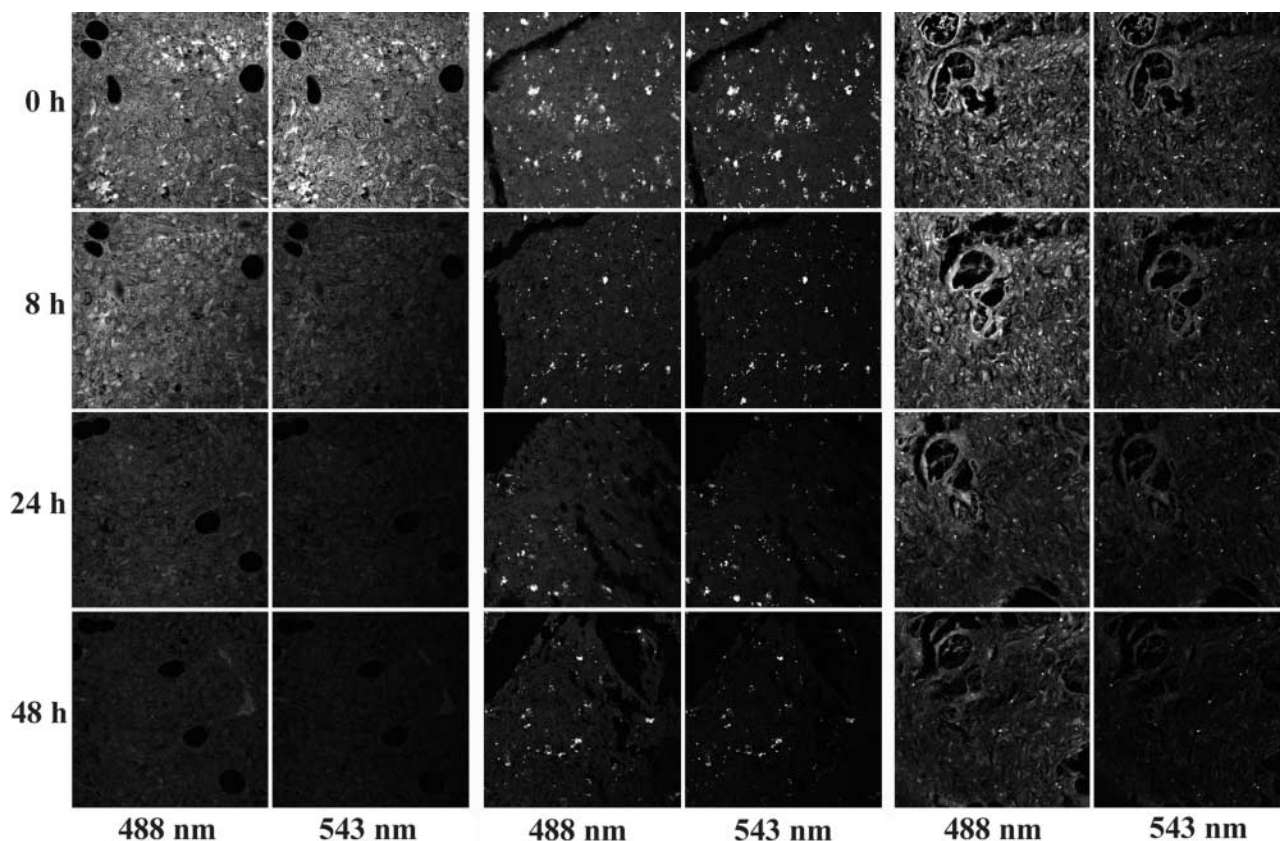


Figure 1 Photographs of irradiated and non-irradiated tissue sections. Left, mouse liver; middle and right, human brain tumor tissue.

FITC-conjugated rabbit anti-mouse (Jackson ImmunoResearch, West Grove, PA, 1:150 for 20 hr). For cooling, several small plastic containers filled with frozen water were placed inside the irradiation box. At regular time intervals the containers were replaced by recently frozen ones.

For image acquisition a confocal scanning laser microscope (LSM 410 inverted; Zeiss) was used. The settings for contrast, brightness, scanning time, and pinhole were identical for each picture in each individual set. Fluorescence emission was recorded through a PLAN-NEOFLUAR $\times 40$, NA 1.3 oil objective (Zeiss; Oberkochen, Germany).

All measurements were made with the following filter sets: chromatic beamsplitter FT 510, bandpassfilter BP 515–565 for argon laser irradiation at 488 nm and FT 560 longpass filter LP 570 for helium neon laser irradiation at 543 nm.

During irradiation the autofluorescence reduces dramatically with time. Most of the structures lose their ability to fluoresce after 24 hr of treatment. Some structures are less affected by the photobleaching and were still visible after 48 hr of irradiation (Figure 1). However, by using a mercury arc lamp (HBO 103) combined with the Plan-NEOFLUAR objective $5\times$

NA 0.15 (Zeiss), all autofluorescence in the illuminated area could be eliminated after 20 min (Figure 2).

After a longer period of irradiation without cooling, the quality of staining for DNA by ethidium bromide and acridine orange was very poor. With cooling during the photo treatment, no difference of the staining quality between treated and untreated sections

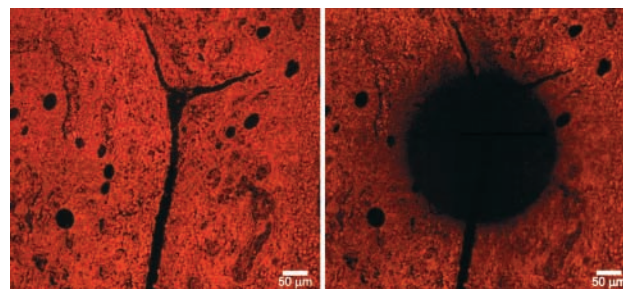


Figure 2 Mouse liver section without irradiation (left) and after 20-min irradiation by a mercury arc lamp (HBO 103) through a Plan-NEOFLUAR-objective $5\times$ /NA 0.15. For image acquisition a digital camera (Polaroid DMC1) and a filter set with excitation 510–560 nm, a chromatic beamsplitter FT580, and a longpass filter LP 590 were used.

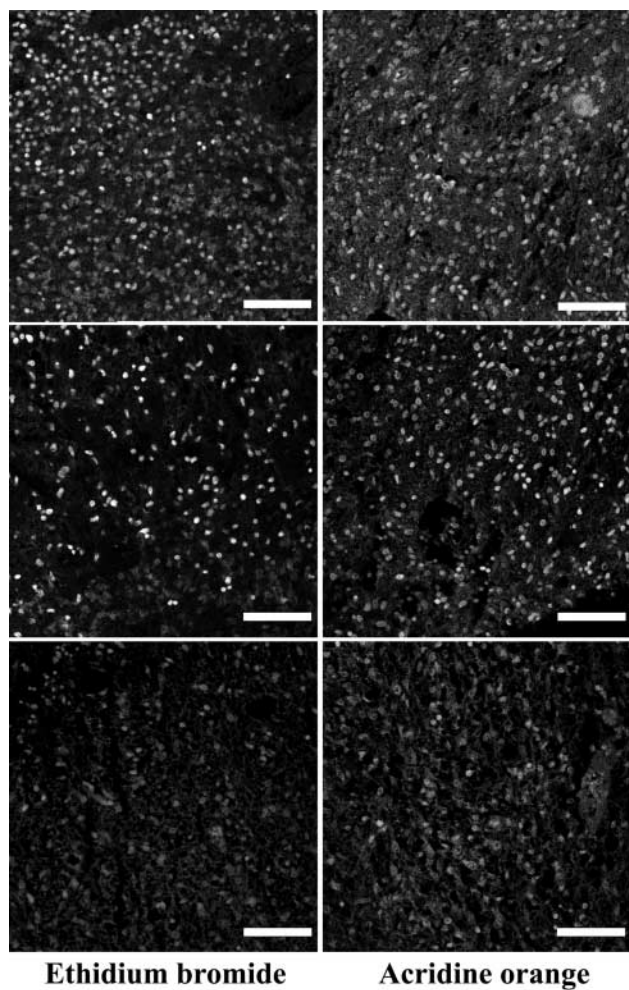


Figure 3 Staining of cell nuclei by ethidium bromide (excitation 543 nm) and acridine orange (excitation 488 nm) in human brain tumor tissue. Top, without irradiation; middle, 48-hr irradiation with cooling; bottom, 48-hr irradiation without cooling. Bars = 50 μ m.

could be detected (Figure 3). The same results were obtained for immunohistochemistry. With cooling, no decrease in the staining quality could be detected (Figure 4).

By illumination with visible or long-wavelength UV light, strong reduction of almost all autofluorescence is possible without adversely affecting the possibility of labeling the tissue with fluorescent markers, thus improving the quality of staining. The results for the human brain tumor tissue used in this study are better than those obtained on the same tissue with CuSO_4 in ammonium acetate buffer or Sudan Black B in 70% ethanol (Schnell et al. 1999), NaBH_4 (Clancy and Cauller 1998), or Pontamine Sky Blue (Cowen et al. 1985). By using fluorescent tubes with a limited window for the emitted wavelength, a chosen range of wavelength could be freed of autofluorescence while another stayed in the original state. This could be of interest when the fluorescent structures give additional information. By use of more than one tube for a special wavelength, the irradiation time can be decreased.

Literature Cited

- Clancy B, Cauller LJ (1998) Reduction of background autofluorescence in brain sections following immersion in sodium borohydride. *J Neurosci Methods* 83:97–102
- Cowen T, Haven AJ, Burnstock G (1985) Pontamine Sky Blue: a counterstain for background autofluorescence in fluorescence and immunofluorescence histochemistry. *Histochemistry* 82:205–208
- Schnell SA, Staines WA, Wessendorf MW (1999) Reduction of lipofuscin-like autofluorescence in fluorescently labeled tissue. *J Histochem Cytochem* 47:719–730
- Steinkamp JA, Stewart CC (1986) Dual-laser, differential fluorescence correction method for reducing cellular background autofluorescence. *Cytometry* 7:566–574
- Van de Lest CH, Versteeg EM, Veerkamp JH, Van Kuppevelt TH (1995) Elimination of autofluorescence in immunofluorescence microscopy with digital image processing. *J Histochem Cytochem* 43:727–730

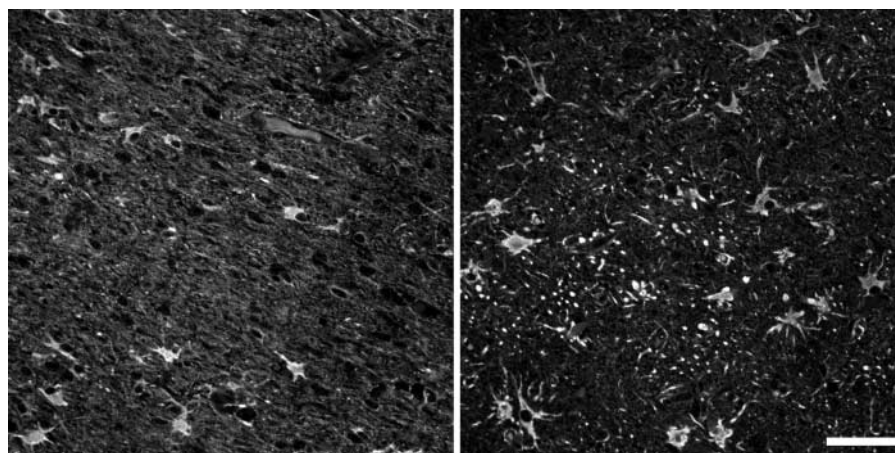


Figure 4 Staining of GFAP with FITC in human brain tumor tissue. Left, without irradiation; right, 24-hr irradiation. The contrast improved without decreasing the labeling quality. Bar = 50 μ m.