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# Note

## Rapid and simple method of photobleaching to reduce background autofluorescence in lung tissue sections

B Santhosh Kumar[1], S Sandhyamani[1*], Shaiju S Nazeer[2] and R S Jayasree[2]

[1]Department of Pathology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Medical College (PO), Thiruvananthapuram-695 011, Kerala, India
[2]Biophotonics and Imaging Lab, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojappura, Thiruvananthapuram-695 012, Kerala, India

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Autofluorescence exhibited by tissues often interferes with immunofluorescence. Using imaging and spectral analysis, we observed remarkable reduction of autofluorescence of formalin fixed paraffin embedded tissues irradiated with light prior to incubation with immunofluorescent dyes. The technique of photobleaching offers significant improvement in the quality and specificity of immunofluorescence. This has the potential for better techniques for disease diagnosis.

**Keywords**: Autofluorescence, Immunofluorescence, Photobleaching, Anti-nuclear antibody

Autofluorescence exhibited either by intrinsic fluorophores[1,2] or induced by fixatives[3,4] during tissue processing may either mask the fluorescence from molecule of interest or interfere with the emission from fluorescent labels. It is a major challenge in immunofluorescence studies[5]. Histochemicals, such as ammonia-ethanol, sodium borohydride, Sudan black B etc., are generally used to reduce autofluorescence[6-8]. However, these techniques have various drawbacks, mainly reduction in the affinity of antigen to antibody due to presence of the chemicals or dyes. Other existing methods are either computational or mathematical models, which subtract the specific spectra of the fluorescent labels from the broader background autofluorescence; however, such models may cause misinterpretation of the results[9,10].

The present study has been aimed to develop a simple method to minimize autofluorescence in formaldehyde fixed archival tissues using photobleaching by irradiation with light prior to incubation with the fluorescent dye-tagged antibodies.

*Corresponding author
Tel:+91 4712524605 (O)
E-mail: sandhyamani@gmail.com

## Materials and Methods

### Tissue sections

Formalin (10% formaldehyde) fixed, paraffin-embedded archival lung tumor tissues were obtained from Department of Pathology, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST). Institutional Ethics Committee (IEC) of SCTIMST approved the study. Thin sections (5 µm) were cut, mounted on glass slides and divided into four sets. Two sets of sections were deparaffinized using two 10-min washes in xylene and the other two sets were used for irradiation without deparaffinization. The first set of deparaffinized sections was irradiated for different time periods, viz. 5, 10 and 15 min and 1, 12 and 48 h with white light directly from the mercury arc lamp at a distance of 20 cm and the second deparaffinized set exposed to a mercury arc lamp through a 40X objective (with I3 and A filters). The two sets of non-deparaffinized slides were also exposed to light similarly.

After irradiation, the paraffin from the third and fourth sets of sections was removed by two 10-min washes in xylene. All four sets of sections were subjected to fluorescence spectral analysis. Following this, all sections were mounted with coverslips using DPX (dibutyl phthalate xylene) and their fluorescence images were studied microscopically. To test the effect of irradiation on the ability of binding of the fluorescence markers to tissues, additional sections were subjected to above test conditions and stained with either acridine orange (AO) or fluorescent antinuclear antibody (FANA) using standard procedure available elsewhere[11,12]. Spectral and imaging features were characterized and compared.

### Fluorescence instrumentation

Digital images were recorded with a Canon Power Shot S70 camera on a Leica Leitz DMRB fluorescence microscope equipped with a red fluorescence filter (Leica I3–513808) and a blue fluorescence filter (A–513808), both located on a manual wheel. Images were acquired with Zoom Browser EX software using a 40×/NA 0.70 objective

lens (Leica) with an ISO speed rating 50 without flash and excitation with 100 W mercury arc lamp (Leica). Autofluorescence spectra were then collected using Spectrofluorometer Fluorolog-III (Jobin Yvon Inc., Edison, New Jersey) with an excitation or emission slit width of 5 nm and 500 nm/min scan speed. The source of excitation light was a Xenon lamp (450 W), and the excitation wavelengths (280 and 410 nm) were selected using double-grating monochromator. The excitation light was allowed to fall perpendicular to the tissue surface (2 x 6 mm) and fluorescence emission was collected using photomultiplier tube at an angle of 22.5$^{o}$ with respect to the excitation beam. The emission spectra were in the range of 300-450 and 440-750 nm for 280 nm and 410 nm excitations, respectively.

## Results

Microscopic imaging of deparafinized tissues that were photobleached directly by mercury arc lamp at 20 cm distance revealed that the autofluorescence of cellular and extra cellular components like cytoplasm, collagen, elastin, etc. reduced dramatically and rapidly with time of exposure (6 to 48 h). Irradiation of tissue sections for 5 min to 3 h did not show appreciable reduction of autofluorescence. After 48 h of exposure, autofluorescence in the tissue section was reduced to 30% (Fig. 1B) of that seen in non-exposed tissues (Fig. 1A). Reduction in autofluorescence of different tissue components varied with time of exposure.

However, when a combination of mercury arc lamp with fluotar objective 40X (NA 0.70) was used, autofluorescence of all cellular and extracellular structures particularly cytoplasm, collagen and elastin in the illuminated area could be eliminated completely within 15 min of irradiation. This photobleaching (Fig. 1C and D) was more rapid using filter A (UV light) than with filter I3 (Blue light).

Acridine orange fluorescence intensity was the same for photobleached and unbleached sections (Fig. 1E and F), indicating that the affinity of the tissue to this dye was unchanged. The results of the fluorescent antinuclear antibody test (Fig. 1G and H) revealed that while irradiation reduced the background fluorescence, it did not affect the binding of tissue by fluorescent markers, thus improving the quality of this staining technique.

Fluorescence spectral results revealed only 60% reduction in the autofluorescence after 48 h of irradiation with mercury arc lamp (Fig. 2) at 410 and 280 nm excitations. We also observed that as the time of light exposure to tissues increased, the intensity of autofluorescence decreased. It was interesting to note that peaks at 560 nm and 665 nm were removed completely after irradiation. Inset of Fig. 2 shows the reduction in autofluorescence with time of exposure under 280 nm excitation which was similar to results using 410 nm excitation.

All non-deparafinized sections showed no reduction in autofluorescence even after 48 h of irradiation directly with mercury arc lamp or in

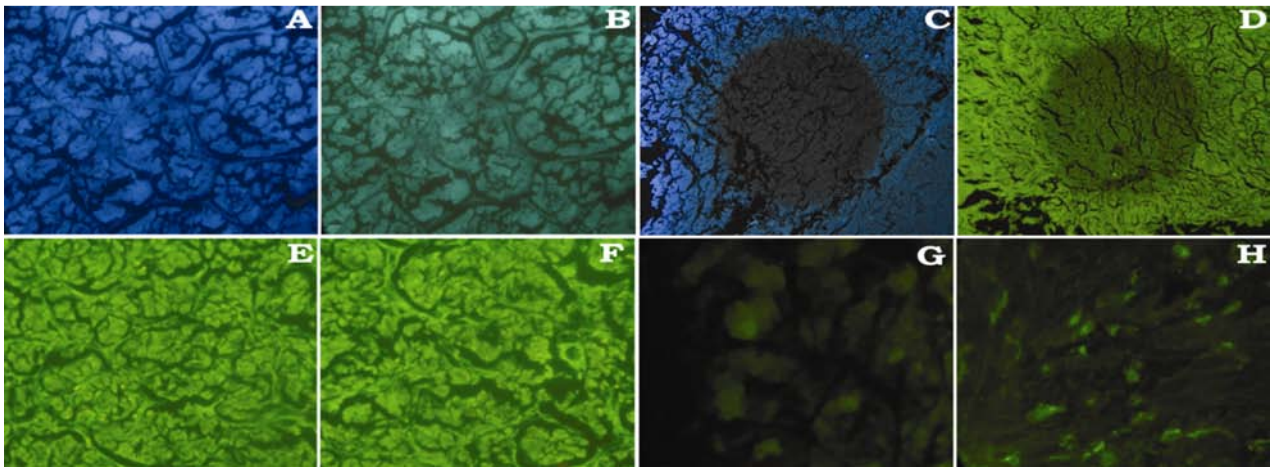


Fig. 1— A & B: Shows autofluorescence of unbleached sections (A) and reduced autofluorescence of photobleached sections using mercury arc lamp at a distance of 20 cm (B); C & D: UV excitation shows greater photobleaching (C) compared to blue excitation (D) (Photobleaching using combination of mercury arc lamp, 40X Fluotar objective and filter); E & F: Acridine orange staining doesnot show any difference between non-bleached (E) and photobleached (F) sections; G & H: Compared to non-bleached section (G) the photobleached section (H) shows sharp and specific anti nuclear antibody staining

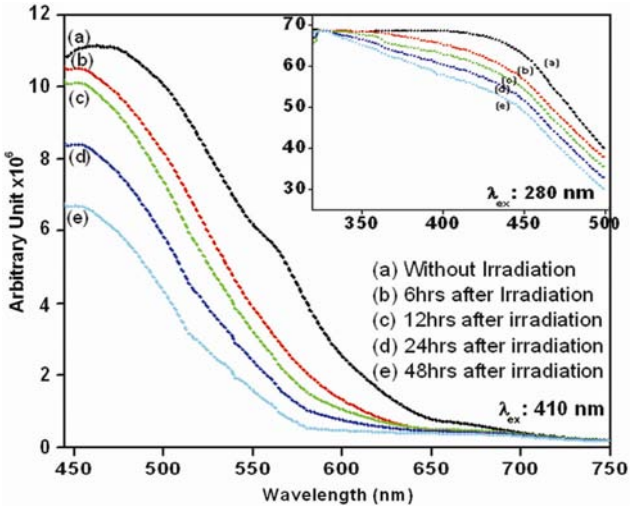


Fig. 2—Spectrum at 410 nm excitation shows a decrease in the fluorescence intensity with increase in time of exposure using mercury arc lamp [Inset shows the spectrum at 280 nm excitation]

combination with the 40X objective and I3 and A filters. This suggested that deparafinization is needed before irradiation.

## Discussion

Autofluorescence is particularly annoying in immunofluorescence[6,7] and other fluorescence techniques, such as *in situ* hybridization and fluorescence quantification studies[13,14]. Non-specific autofluorescence signals can mask the real fluorescence/immunofluorescence signals from the specific markers and result in false positive or false negative reporting. Here, we report a simple method that reduces most types of autofluorescence in tissue sections without adversely affecting the staining of tissues with fluorescent markers. This study showed that photobleaching with a combination of mercury arc lamp and 40X objective of a microscope and specific filters could dramatically and rapidly reduce the autofluorescence of tissue sections within 15 min.

We observed that after 48 h of direct photobleaching of sections at a distance of 20 cm from the mercury arc lamp reduced the autofluorescence by almost 80%. The photobleaching rate of different biological components was different, which might be due to the photoresistance/ photosensitivity of various components in the sections. However, the above mentioned combination used by us was effective in eliminating all the autofluorescence within 15 min, without affecting the fluorescence emission from the subsequently added stains, such as AO and FITC used in the anti-nuclear antibody (ANA) immunofluorescence technique. This significantly decreased the background autoflorescence to produce a better contrast and hence improved the quality of the immunofluorescence techniques. In our study, more photobleaching was observed with filter A (UV) than filter I3, which might be attributed to the higher energy of the UV light than the blue light.

During spectroscopic measurement of tissue sections, we observed a definite decrease in the autofluorescence intensity with increasing time of exposure. As found with imaging study, during spectral characterization, we also observed that peaks at 560 nm and 665 nm were more susceptible to photobleaching after 6 h of irradiation. This suggested that those molecules which emitted at 560 nm and 665 nm photochemically changed their electronic configuration, leading to the quenching of fluorescence emission. In addition, we observed that autofluorescence decrement was only 60% even after 48 h of irradiation. Thus, for studies based on spectroscopic measurement, the photobleaching techniques may not be very useful in reducing background autofluorescence.

An interesting phenomenon observed was that the tissue sections containing paraffin wax did not get bleached even after 48 h of irradiation. This might be due to protective effect of paraffin as observed in an earlier study[15].

In conclusion, photobleaching using a combination of mercury arc lamp, 40X microscope objective and appropriate filters is a simple and rapid method to reduce intensity of autofluorescence in tissues and other biological molecules, while preserving the specificity for fluorescent labels. Further studies are being done using different types of tissues, various fixatives and wavelengths of excitation light to exclude autofluorescence in secondary fluorescence studies. Our earlier study has shown that different substrata have varying degree of autofluorescence[16]. A combination of a substratum with least autofluorescence and the present technique is expected to produce more contrast and sharper images for fluorescence studies.

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