

# Short Technical Reports

## Photobleaching of Arterial Autofluorescence for Immunofluorescence Applications

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### ABSTRACT

*Immunohistochemical localization of low-level antigens in the arterial vasculature is complicated by the presence of complex molecules such as collagen, elastin, cholesterol, and fluorescent lipids that exhibit autofluorescence over a wide spectrum of wavelengths. UV irradiation of arterial vasculature has remained ineffective in preparing samples for immunofluorescent staining because of the recovery of the endogenous fluorescence within a short time following treatment. Therefore, we sought to further enhance the signal-to-noise ratio in arteries by optimizing the photobleaching of this tissue. We report here that the use of filtered sunlight significantly reduces arterial autofluorescence compared to standard UV shortwave and longwave irradiation and maintains multiple antigen epitopes suitable for immunohistochemical analysis. Using this method, we localized low-level laminin-5 isoform expression in situ, which was previously indistinguishable from endogenous autofluorescence.*

### INTRODUCTION

Endogenous fluorescent molecules containing multiple tissue fluorophores complicate the use of immunofluorescence labeling in tissues such as the arterial vasculature, especially when attempts are made to localize low-abundance antigens in this tissue. In addition to elastin and collagen, cholesterol, fluorescent lipids, and other compounds further complicate spectral analysis of this tissue (2). Enzymatic amplification of the specific fluorescent signal above background is one means of overcoming this problem. For example, the use of a fluorogenic phosphatase substrate, 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-[3H]-quinazolinone, otherwise known as the ELF substrate (7), alone has improved sensitivity in several circumstances.

The ELF substrate produces a photostable precipitate upon dephosphorylation that amplifies antibody detection of low-abundance antigens in immunohistochemical analyses. This dephosphorylation is accomplished by alkaline phosphatase conjugation to secondary antibodies via a streptavidin-biotin bridge. The ELF technique has been used to successfully localize low-level expression of  $\beta$ -galactosidase fusion proteins in yeast, overlapping gene expression regions in mouse embryos, and adult zebrafish retinal tissue antigens (1,4,6). Although ELF alone significantly improves sensitivity in several tissues, its effect cannot overcome arterial autofluorescence. Hence, we tested several different conditions to determine which had the greatest reduction in tissue arterial autofluorescence.

Using defined conditions of filtered sunlight to decrease autofluorescence in conjunction with signal amplification using the ELF substrate, we observed low-level antigen localization that was distinguishable from endogenous autofluorescence and that the antigen epitopes are recognized by specific monoclonal antibodies. Using specific monoclonal antibodies, we demonstrated the conservation of multiple antigenic epitopes following this treatment. These data provide an effective technique to increase the sensitivity of immunofluorescence staining by the reduction of background interference.

### MATERIALS AND METHODS

#### Tissue Preparation

Adult aortic sections for initial studies were obtained from Roy C. Smith (St. Elizabeth's Medical Center, Boston, MA, USA). All subsequent animals were maintained by Animal Care Facilities at the University of Nevada, Las Vegas according to protocol R701-1297-136: Developmental Studies in Rats and Mice. Adult rat tissues were fixed at 4°C for 24 h in each of three successive paraformaldehyde solutions (4% w/v) containing 5%, 10%, and 15% sucrose, respectively. Specimens were frozen in Miles Tissue-TEK OCT 4583 embedding medium using standard cryomolds and liquid freon. Tissue sections (8  $\mu$ m

thick) were cut using a Microtome Cryostat HM 505 E (Microm GmbH, Walldorf, Germany) and adhered to poly-L-lysine (0.8 mg/mL)-coated glass slides for photobleaching and staining.

#### Photobleaching

Control tissue sections were maintained at 25°C without direct contact to solar exposure. UV-irradiated tissues were maintained at 25°C, 5 cm from a UV longwave (365 nm) or UV shortwave (254 nm) illuminator. For treatment with filtered sunlight, tissues were maintained at 27°C in a greenhouse at constant oxygen (20%) and carbon dioxide (5%) concentrations. Each day constitutes 11.98–12.24 h of solar exposure.

#### Solar Filter

Greenhouse glass consists of two panes with special coating. The inner surface of the outermost pane is coated with darkglass, isolated to have a better k-value, which decreases heat loss. The inner pane is coated with an oxid-layer (tin oxide) that influences the penetration of radiation. This system has a light transmission above 50%–55% for the visible part of the spectrum (380–780 nm) and 30%–32% transmission in the UV spectrum (10–400 nm).

#### Antibodies

Primary monoclonal antibody (mAb) to all three subunits of laminin-5 (CM6,  $\alpha$ -3 subunit; FM3,  $\beta$ -3 subunit; TR1,  $\gamma$ -2 subunit) were generously provided by Desmos (San Diego, CA, USA). Goat anti-rat elastin polyclonal antibody (pAb) 4060-1104 and goat anti-rat collagen type 1 pAb 2150-1908 were obtained from Biogenesis (Kingston, NH, USA). Secondary antibodies were biotin-labeled donkey anti-goat (H+L) conjugate RDI-705065147 obtained from Research Diagnostics (Flanders, NJ, USA) and biotin-SP goat anti-mouse IgG (H+L) conjugate B2763 (2 mg/mL) obtained from Molecular Probes (Eugene, OR, USA).

#### Immunohistochemistry

Tissues were prepared according to

Molecular Probes ELF<sup>®</sup>-97 Immunohistochemistry Protocol MP 06600. All reactions were performed in a humidified chamber at 25°C. Tissue sections were incubated with blocking buffer (30 mM Tris-HCl, 150 mM NaCl, 1.0% bovine serum albumin, 0.5% Triton<sup>®</sup> X-100, pH 7.5), followed by incubation with primary antibody (1:500 dilution in wash buffer: 30 mM Tris-HCl, 150 mM NaCl, pH 7.5). Subsequent incubations with biotinylated secondary antibody (1:1000 dilution in wash buffer), streptavidin (1:250 dilution in wash buffer), and ELF-97 Substrate Reagent (1:20 dilution with ELF-97 Immunohistochemical Reaction Buffer) were performed after serial tissue incubations with wash buffer. The reaction was arrested using stop buffer (25 mM EDTA, 1.0 mM levamisole, 0.05% Triton X-100 in 1× PBS, pH 8.0) and mounted in ELF-97 Immunohistochemical Mounting Medium (Molecular Probes).

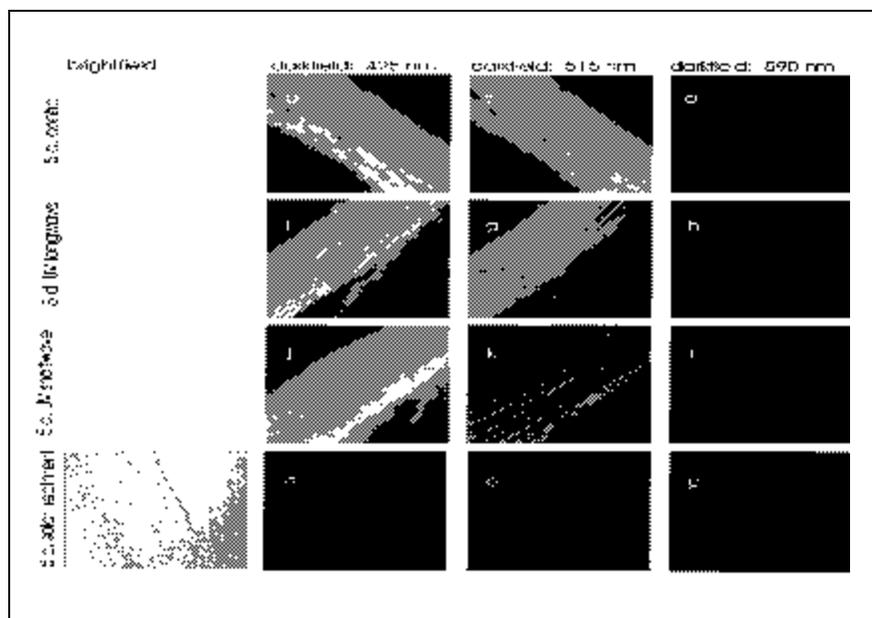
### Microscopy

Stained tissues were visualized using a Leica DM LB (Leica GmbH, Wetzlar, Germany) immunofluorescence microscope equipped with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) long-pass and fluorescein-5-isothio-

cyanate (FITC) filter sets. ELF substrate excitation and emission wavelengths are 360 and 535 ± 18 nm, respectively. Images were collected using SPOT Diagnostic digital imaging photographic equipment in bright and darkfield. UV band pass excitation filter (340–380 nm) and long pass suppression filter (425 nm) were used for the collection of DAPI images; 450–490 band pass excitation filter and 515 long pass suppression filter were used for the collection of FITC images; 515–560 band pass excitation filter and 590 long pass suppression filter were used for the collection of rhodamine images. Quantitation of relative autofluorescence was measured with Adobe<sup>®</sup> Photoshop<sup>®</sup> imaging software using Image analysis.

### RESULTS

To remove the primary autofluorescence of endogenous molecules, we performed multiple photobleaching techniques to determine a method of reducing tissue fluorescence. Prolonged irradiation of arterial tissue with UV long pass (Figure 1, e–h) and UV short pass (Figure 1, i–l) filters reduced the intensity of autofluorescence only marginally, whereas exposure to filtered so-



**Figure 1.** Fluorescent micrographs taken in brightfield and UV (425, 515, and 590 nm) after five days of control, shortwave, longwave, or solar radiation exposure. (a–d) Control sample. (e–h) UV longwave sample after five days of exposure. (i–l) UV shortwave sample after five days of exposure. (m–p) Solar-treated sample after five days of exposure.

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lar radiation (Figure 1, m–p) diminished the endogenous autofluorescence of arterial vascular tissue significantly compared to UV-treated and control (Figure 1, a–d) tissue sections.

Control tissues autofluorescence experienced nominal photobleaching from the photomicroscopy procedure by approximately one-fifth at 425, 515, and 590 nm (Figure 2A). UV long pass did not reduce autofluorescence emissions at 425 nm but did reduce emissions at 515 nm, by 58% at 515 nm, and by 9% at 590 nm (Figure 2C). UV shortwave reduced emissions at 425 nm by 13%, by 30% at 515 nm, and by 25% at 590 nm (Figure 2B). Solar-treated tissue sections experienced the greatest overall level of irreversible photobleaching, reducing emissions by 73% and 77% at 425 and 515 nm, respectively, but did not reduce emissions at 590 nm (Figure 2D).

Epitopes for collagen I, elastin, and laminin-5 were resistant to photodegradation and photochemical oxidation damage during filtered solar radiation exposure. Monoclonal antibodies to all three chains of the laminin-5 isoform (Figure 3, d and e) and polyclonal antibodies to collagen I (Figure 3f) and elastin (Figure 3c) bound their ligands with sufficient affinity for immunohistochemical applications. The reduction of endogenous autofluorescence (Figure 3a), and the preservation of these antigens allowed for the detection of these proteins by both traditional and enzymatically enhanced variations of immunohistochemistry.

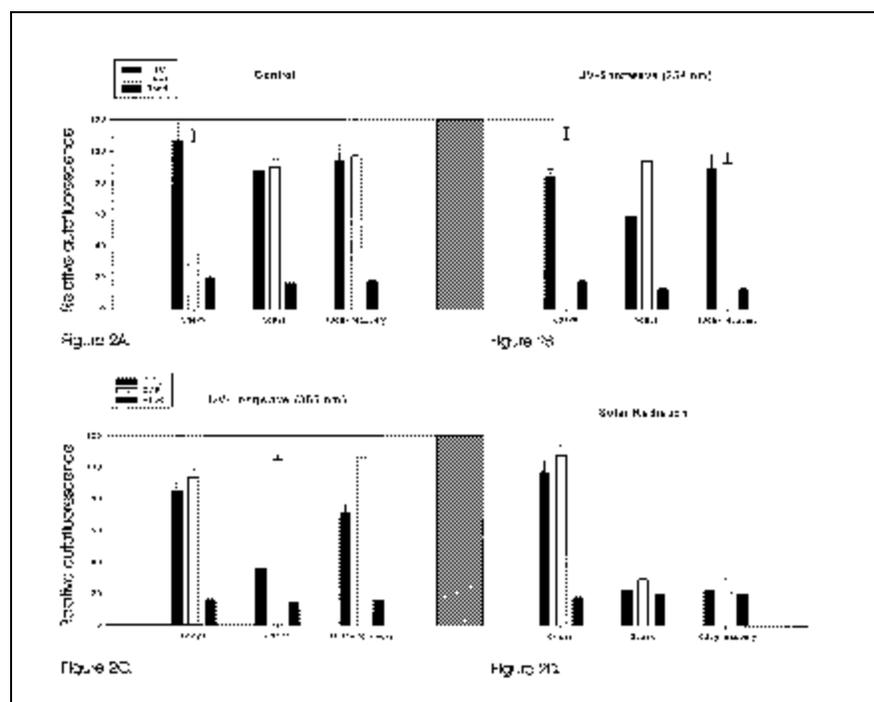
## DISCUSSION

Our results suggest that filtered solar radiation is a simple and effective method for reducing arterial tissue autofluorescence. The effect of filtered solar radiation on emissions at 425 and 515 nm are at least 20% better than both UV shortwave and UV longwave radiation. We observed an 84%–100% recovery of autofluorescence 10 days after conventional photobleaching with UV longwave and UV shortwave filters. In contrast, we showed that a five-day exposure to filtered sunlight significantly reduced autofluorescence, which does not increase 10 days after treat-

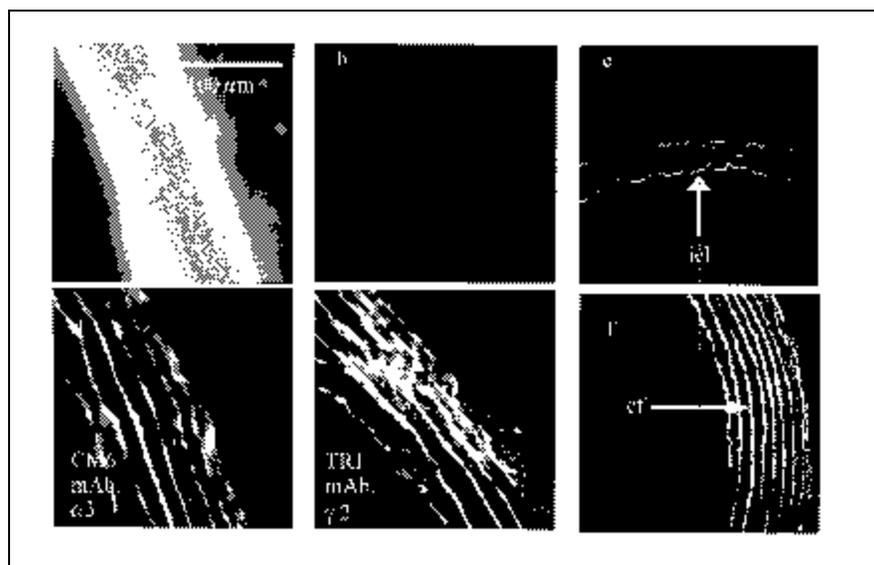
ment cessation, remaining at 27% (425 nm) and 23% (515 nm) of the original signal after the 10-day recovery period.

The photobleaching of these compounds may be caused by a chemical change of the excited fluorophore into a

species that cannot fluoresce because of the inability to further absorb excitation photons. Thus, sustained, filtered-solar radiation may provide the multiple wavelengths required to yield photochemical alterations of these complex



**Figure 2.** Graphs representing relative amounts of photobleaching and recovery for each condition over the specified time.  $n = 8$  measurements taken per sample.



**Figure 3.** Tissues before and after treatment with solar radiation followed by staining show preserved epitope integrity. (a) Autofluorescence in untreated cross-section. Treated samples labeled using (b) normal mouse IgG, (c) polyclonal anti-elastin with rhodamine-conjugated secondary Ab, (d) monoclonal anti- $\alpha 3$  subunit of laminin-5, visualized with ELF-97, (e) monoclonal anti- $\gamma 2$  laminin-5, visualized with ELF-97, and (f) polyclonal anti-collagen with FITC-conjugated secondary Ab.

and varied molecules to either a lower or absent fluorescence state, a result not typically accomplished by traditional laser and UV radiation techniques.

The difference in autofluorescence recovery between these treatments was likely due to the relative reduction of autofluorescent compounds by neighboring electron donors in the tissue. Exposure to filtered sunlight provides excitation over a broader spectrum of wavelengths than either UV longwave or UV shortwave bands and thus may cause a more effective oxidation of fluorescent compounds and neighboring molecules. This is reflected by the rate of recovery of autofluorescence following photobleaching: 10 days after a five day photobleaching period, autofluorescence at 425 nm returned to 100% and 84% of pre-bleached levels in tissues exposed to UV shortwave and UV longwave spectra, respectively, while no recovery of autofluorescence was observed in tissues exposed to filtered sunlight after the same time course.

Photobleaching may affect each fluorescent compound of the arterial wall differently. Complex autofluorescent molecules such as collagen and elastin contain more than one fluorophore that may photobleach at different wavelengths (3,5). The intensity of collagen autofluorescence (515 nm) decreased significantly (Figure 1, c, g, k, and o) more than that of elastin autofluorescence (425 and 515 nm) (Figure 1, b, f, j, and n), suggesting that photobleaching affects each compound of the arterial wall to different extents.

In addition, our results show that despite any effects solar radiation induces in the autofluorescence of the sample, multiple binding epitopes are preserved. This allows more sensitive immunohistochemical staining for non-abundant proteins because solar exposed samples are both lower in background and have uncompromised epitopes.

This method indicates that there are variations in the time and wavelength dependence of fluorescence decay among fluorescent molecules in this tissue. Given these considerations, a photobleaching method appropriate for the tissue and immunofluorescent chromophore can be determined; double and triple labeling immunohistochemistry experiments involving two or

more chromophores may benefit from this method of tissue preparation. These results can be replicated in a variety of greenhouse glass-enclosed environments based on the need for autofluorescence reduction across multiple wavelengths. The use of rhodamine may not require photobleaching in this tissue, whereas the use of DAPI and FITC may require intense methods of photobleaching.

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