Histochem Cell Biol (1996) 106:587–592

ORIGINAL PAPER

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Fluorescence and confocal laser scanning microscopy imaging of elastic fibers in hematoxylin-eosin stained sections

Accepted: 28 August 1996

Abstract We have studied the possibility of associating fluorescence microscopy and hematoxylin-eosin staining for the identification of elastic fibers in elastin-rich tissues. Elastic fibers and elastic laminae were consistently identified by the proposed procedure, which revealed itself to be easy and useful for the determination of such structures and their distribution. The fluorescence properties of stained elastic fibers are due to eosin staining as revealed by fluorescence analysis of the dye in solution, with no or only minor contribution by the elastin autofluorescence. The main advantage of this technique resides in the possibility of studying the distribution of elastic fibers in file material without further sectioning and staining. The use of the confocal laser scanning microscope greatly improved the resolution and selectivity of imaging elastic fibers in different tissues. The determination of the three-dimensional distribution and structure of elastic fiber and laminae using the confocal laser scanning microscope was evaluated and also produced excellent results.

Introduction

Eosin is a very common plasmal stain. It is acidic and usually employed in combination with basic dyes such as azures and hematoxylins. Eosin has long been recognized as a green fluorescent dye (Pearse 1972) and was previously used as a fluorescent protein tracer (Chadwick et al. 1958). Currently, it is used in protein motion and association studies using fluorescence spectrophotometry (Kenny et al. 1993; Londo et al. 1993).

Tissues stained with eosin alone show very diffuse fluorescence but, recently, it was proposed that eosin Y fluorescence in Giemsa-stained tissue sections could be used to discriminate calcified bone and elastic fibers using the fluorescence microscope (Bradbeer et al. 1994). The selective fluorescence exhibited by these two main tissue components is based on the very faint eosin staining, while intensely eosinophilic substrates and those stained with the highly absorbing basic dyes show no fluorescence or just a very faint signal.

Considering the possibility of using eosin fluorescence for the selective identification of some tissue components, we have extended this approach to the study of elastic fibers in hematoxylin-eosin (H&E) preparations. This paper then describes the results of observations with the fluorescence microscope of some H&E stained sections and evaluates the use of the confocal laser scanning microscope to image elastic fibers in the same preparations. Furthermore, we have also studied absorption and fluorescence spectra of eosin and phloxine, a closely related dye, in solution, to correlate them with the fluorescence detected in tissue sections.

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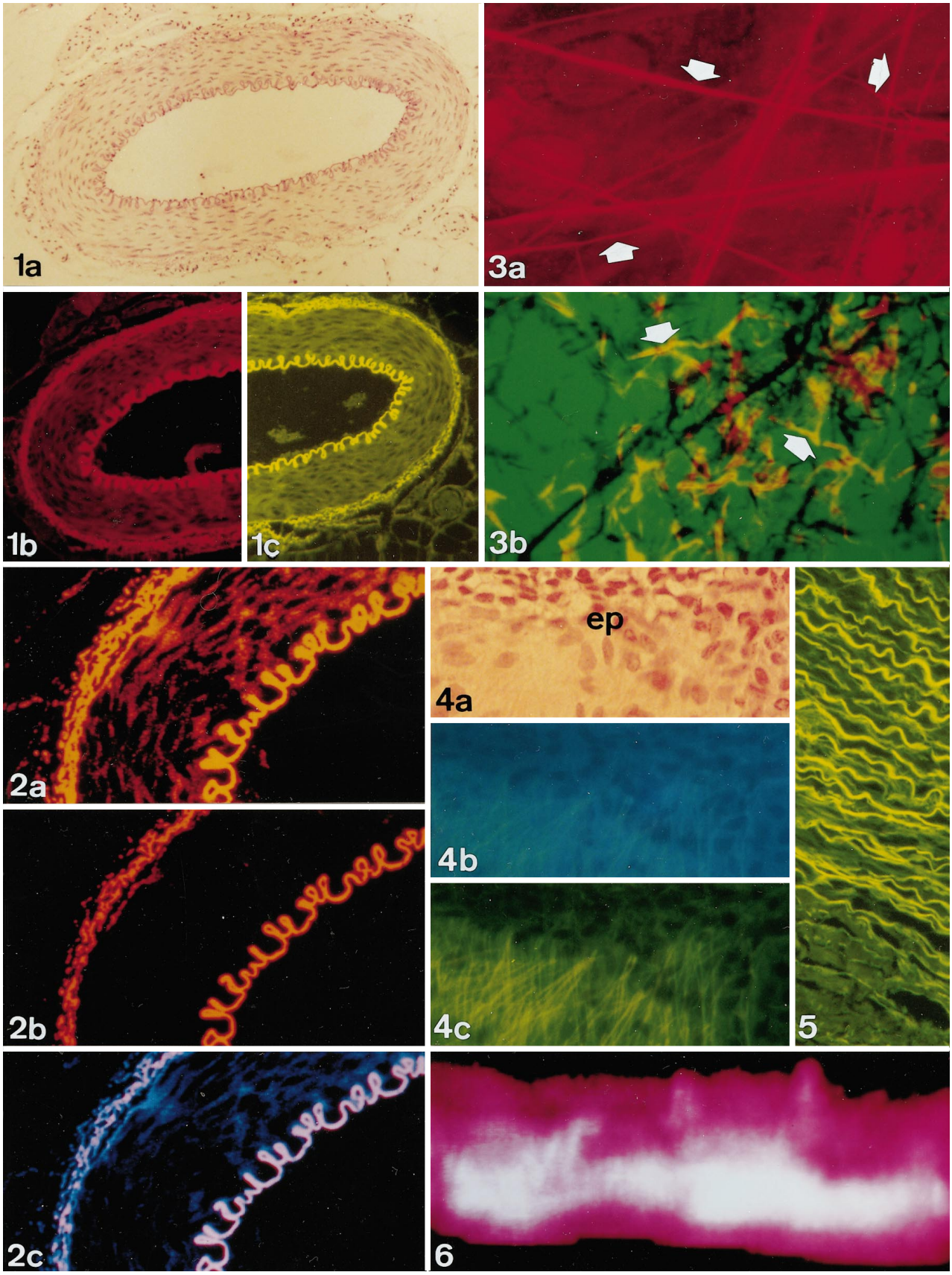
Materials and methods

File material was used throughout this study. Mainly human and other mammalian paraffin tissue sections were used, regardless of the fixation procedure employed. H&E preparations from at least three research and diagnosing centers were utilized. The best results were achieved by combining Harry's hematoxylin plus eosin/phloxine staining procedure, as follows: Harry's hematoxylin for 4 min followed by differentiation in 70% ethanol:1 N HCl (9:1, v:v) and washing in running tap water for 10 min plus eosin:phloxine (10:1; v:v)(1% solution of each) in acidified ethanol for 2 min. The mounting medium was Canada balsam. Erlich's hematoxylin plus alcoholic eosin stained material produced very poor results.

A Zeiss Axioskop miscroscope operating with reflected light was used in this study. Fluorescence of H&E stained sections was examined with the following excitation/emission filter combinations: 395 nm and a broad band pass barrier filter, 420/530 nm, and 530/600 nm, for blue, green, and red fluorescence, respectively. Alternatively, analyses were carried out using a Zeiss laser scanning microscope model LSM310, equipped with an argon laser emitting visible light at 543 nm, for the analysis of the eosin/phloxine red fluorescence. Plan-Aplochromatic 20× and 40× dry and 40× (NA=1.4) oil immersion objective lenses were used. Confocal analyses were carried out under different work condi-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tions, which are described in the figure legend when essential for the image comprehension. A general procedure was the use of a relatively large pinhole setting (usually 60) for attaining better imaging of long fibrillar elements. Images were obtained as gray levels and artificially colored as red, green or blue, using the RGB system of the controlling software. Since similar results were achieved by analyzing the green fluorescence, the analyses were restricted to results from the red fluorescence.

Fluorescence analyses were carried out in a Hitachi F-3010 fluorescence spectrophotometer working with a Xe lamp. Scanning velocities ranged from 5 to 10 nm/s, using solutions of each dye in water and in absolute ethanol. Emission and excitation spectra were recorded for diluted solutions and the effects of concentration on emission spectra were also investigated.

Results

The materials presented here are very common in histology and, for the sake of brevity, we will concentrate on the description of imaging results rather than the distribution of elastic fibers. It is also worth mentioning that mounting in Canada balsam did not interfere with the fluorescence analyses in our preparations.

Figure 1a shows a muscular artery of the rat jejunal submucosa, as seen after H&E staining. Figures 1b,c corresponds to the same material as observed under fluorescence microscopy using filter sets for red and green fluorescence, respectively. The conspicuous internal elastic lamina of the intima and thin fibers of the adventitia are resolved under these conditions. The two fluorescence filter sets result in virtually identical results, except that red fluorescence is visually less intense.

Figure 2 depicts the same section as seen in the previous figures but imaged with the confocal laser scanning microscope. Figure 2a corresponds to the non-confocal mode and is thus similar to Fig. 1b,c. Figure 2b is the confocal imaging of the same region using the confocal mode. Figure 2c corresponds to the superpositioning of Fig. 2b over Fig. 2a, which was taken in the blue channel. This thus demonstrates that confocal imaging allows a better resolution of elastic fibers.

Figure 3a is an aspect of the mouse mesentery as observed under the fluorescence microscope with the filter set for red fluorescence. Elastic fibers are easily observed but are poorly distinguished from the background. Figure 3b illustrates the use of the confocal laser scanning microscope, in which elastic fibers were optically imaged and superpositioned to the phase contrast image previously taken in the green channel. Since elastic fibers present in just one optical section are represented, this results in apparently fragmented profiles.

Figure 4a shows an H&E stained section of the rabbit larynx and Fig. 4b,c represents the same section region as seen under the filter set for blue and green fluorescence, respectively. In Fig. 4b, elastic fibers are seen as greenish-silver structures and in Fig. 4c they are easily observed in green.

Figure 5 was taken from a human aorta cross section after H&E staining and imaged with the fluorescence microscope using the filter set for green fluorescence. Elastic lamellae are long and continuous in the media and short in the adventitia, clearly depicting different densities and spacial distributions.

Figure 6 is a *z*-view of an elastic lamella such as those seen in the median layer of Fig. 5. Forty optical sections were taken, 0.5 μm apart from each other, reconstructed, and observed *en face*. The thickness of the lamina thus corresponds to the section thickness, i.e., 20 μm. Regardless of their origin, details of the surface are delineated.

Figures 7 and 8 show sections of the human skin stained by Weigert's resorcin-fuchsin technique (Weigert 1898) and H&E plus confocal laser scanning microscopy, respectively. These two figures demonstrate that some of the elastic fibers fuse together and form thick and flat structures, assuming different aspects, which are clearly revealed by both methods.

Figures 9 and 10 present results of the fluorescence spectrophotometric experiments for eosin and phloxine, respectively. We have employed two conditions, water and absolute ethanol, to verify the possible effect of dehydration on the dye fluorescence. In absolute ethanol, eosin and phloxine fluorescence shows a shift to green and a marked decrease in intensity. The results are summarized in Table 1. Figures 9d and 10d demonstrate the

Fig. 1 **a** Hematoxylin-eosin (H&E) staining of a muscle artery of the rat jejunal submucosa. Despite the relatively distinct appearance of the intimal elastic laminae, no further detail is observed. **b** The fluorescence view of a region of **a** using the filter set for red fluorescence. The intimal elastic laminae is readily seen as well as additional elastic elements of the adventitia. Diffuse staining in the media is due to basement membrane staining of smooth muscle cells. **c** The aspect of the complementary region of **b**, clearly depicting images of the elastic elements. ×116

Fig. 2 **a** The fluorescence exhibited by the same material as Fig. 1a using the non-confocal mode of the confocal laser scanning microscope. **b** A confocal image of a single optical section of the same area as in **a**. High resolution of the elastic elements was achieved. **c** The superimposition of **b** on the same image seen in **a**, which was acquired in the blue channel of the RGB system. ×288

Fig. 3 **a** The fluorescence view of elastic fibers in the mouse mesentery. Elastic fibers are seen but not clearly resolved from the background fluorescence. **b** The superimposition of a single optical section obtained with the confocal laser scanning equipment on the phase contrast image taken in the green channel of the RGB system. Since only one optical section was taken, elastic fibers (*arrows*) appear short and fragmented. ×1460

Fig. 4 **a** An H&E stained section of the rabbit larynx showing part of the epithelium (*ep*) and the lamina propria. Under UV illumination and with a blue barrier filter, elastic fibers show a faint yellowish-green signal. **c** The same field as in the previous figures, now observed with the filter set for green fluorescence. Elastic fiber distribution is clearly observed. ×930

Fig. 5 The green fluorescence of elastic elements in the human aorta after H&E staining. Distinct aspects of the elastic elements in the media and adventitia are clearly revealed. In the adventitia, elastic elements are oblique or perpendicular to the plane of the section and appear as short segments. ×930

Fig. 6 An en face view of an elastic lamella of the rabbit aortic media. Three-dimensional reconstruction of 40 optical sections of an elastic lamella of the rabbit aorta after H&E staining as obtained by the confocal laser scanning microscope. Optical sections were 0.5 μm apart from each other. Surface structure is observed, with evident waving and small projections. ×3100

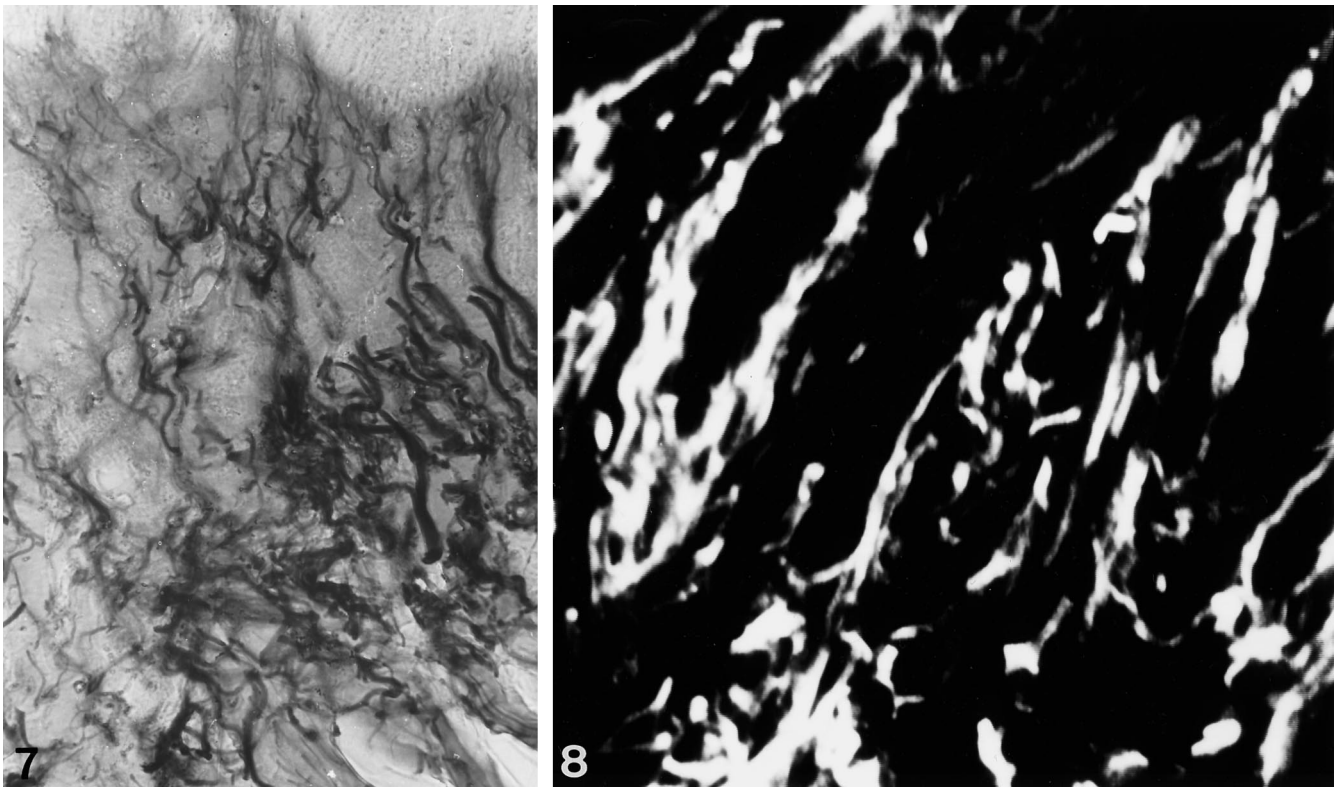

Fig. 7, 8 Human skin. In **Fig. 7** a section of thick human skin stained by Weigert's fuchsin-resorcin is shown. Elastic fibers are thick and commonly fuse together to form flattened structures, which are readily observed with the confocal laser scanning microscopy of an H&E stained section (**Fig. 8**). Details of the elastic fiber distribution and structure are comparable with the two methods. The elastin fluorescence is obviously not observed in the so-called oxytalan fibers. **Fig. 7** ×470, **Fig. 8** ×560

effect of dye concentration on fluorescence emission. In spite of a minor red shift, there is a marked decrease in fluorescence intensity as dye concentration increases.

Discussion

As molecular aspects of elastic fiber structure, biosynthesis and physical properties are revealed, their roles in extracellular matrix biology and pathology gain increasing importance. Furthermore, the possibility of integrating the presence and distribution of elastic fibers with other matrix components in special fibrillar collagens and proteoglycans has allowed for a better understanding of biomechanics in tissues with complex architecture and functioning (Carvalho 1995; Carvalho and Vidal 1995).

The purpose of the present investigation was to demonstrate the usefulness of employing fluorescence microscopy in the identification of elastic fibers in H&E stained sections, rather than analyzing physico-chemical aspects involved with the phenomenon itself or the fiber distribution in specialized tissues. However, the comparison of the fluorescence exhibited by elastic fibers after H&E staining and the fluorescence spectra of eosin and phloxine, and the study of the distribution of elastin in elastic fiber-rich tissues, demonstrated important aspects of both.

It was shown that the fluorescence spectra of eosin in solution and fluorescence aspects of eosinophilic material in histological sections resemble each other. Furthermore, elastic fiber autofluorescence makes little contribution to the fluorescence of elastic fibers in H&E preparations, as investigated in other systems (Carvalho and Taboga 1996).

Eosin and phloxine fluorescence spectra are very similar to each other. Eosin showed an emission peak centered at 550 nm after excitation with 490-nm light. Excitation with 265-nm light resulted in emission peaks at 325, 390, and 550 nm, the latter being around 10 times more intense than the others. This suggests that complex internal energy transitions occur and that most of the emission of the near UV-blue emission is absorbed by other molecular components, resulting in the higher emission at 550 nm. Under the fluorescence microscope, excitation with green light also resulted in red fluorescence. The wide fluorescence spectra detected for both eosin and phloxine strengthens the idea of the occurrence of complex inter- and intramolecular energy transitions.

The effect of dye concentration in solution (Figs. 9d,10d) is probably related to a concentration quenching effect (Pearse 1972), usually resulting from intermolecular energy transitions. This effect is also observed in tissue sections in such a way that intensely eosinophilic structures show very low fluorescence or none at all. In solution, this results in a red shift, which suggests that most of the blue and green fluorescence is ab-

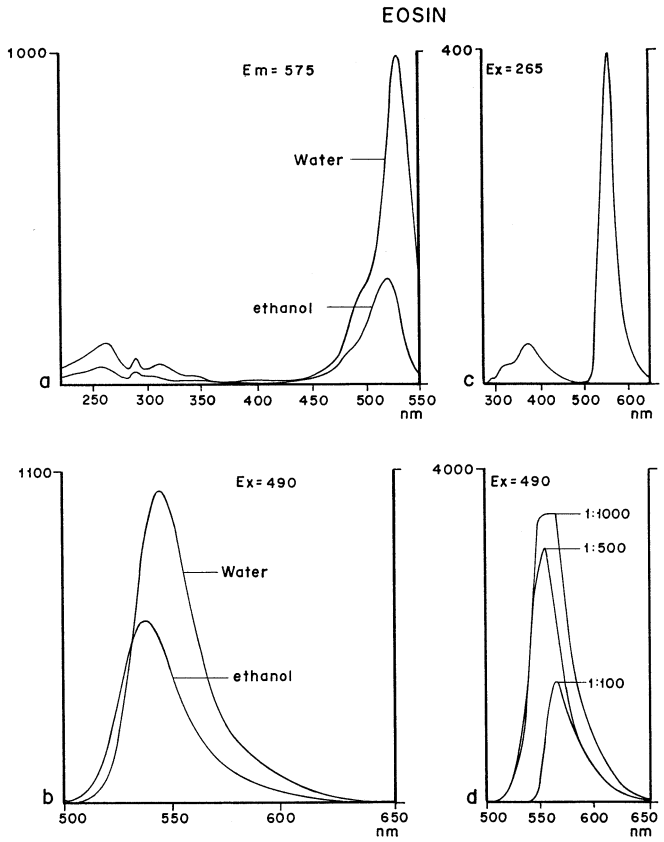

Fig. 9a–d Fluorescence properties of eosin Y in solution. **a** The excitation spectra of 14.5 mM eosin Y in water and absolute ethanol. The fluorescence signals are similar to each other, except for a slight shift of the absorption peak at longer wavelengths and higher intensities in water. **b** The emission spectra of eosin Y after excitation at 490 nm. Emission in water is more intense and shows a slight red shift. **c** The emission spectrum after excitation at 265 nm. Excitation at this wavelength results in a very intense yellowish-green fluorescence. **d** The effect of dye concentration in water. Increasing dilutions result in higher fluorescence intensities. In this case, the dye concentration results also in a red shift. The 1:100 spectrum corresponds to that of 14.5 mM eosin Y solution

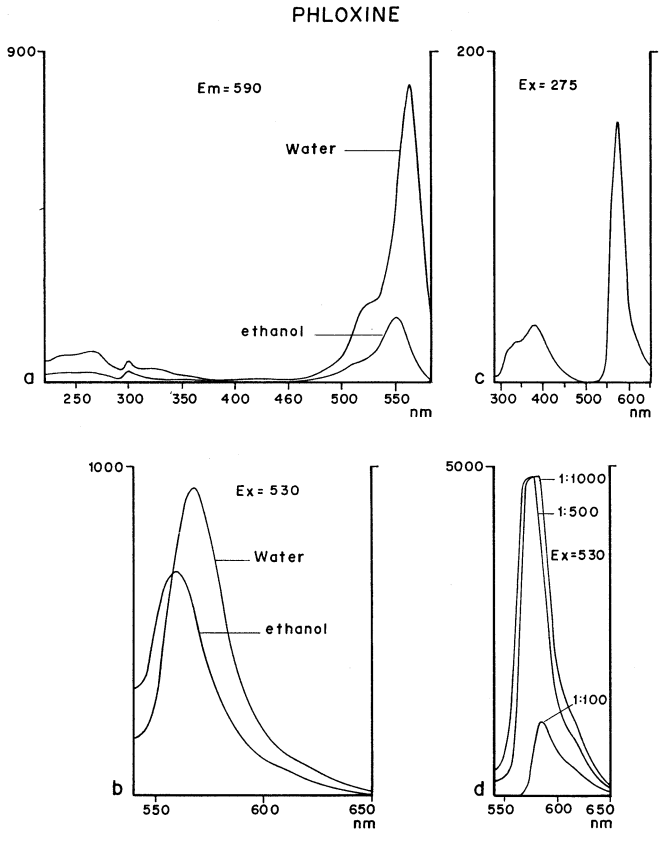

Fig. 10a–d Fluorescence properties of phloxine in solution. **a** The excitation spectra of 12 mM phloxine solution in water and absolute ethanol. The spectra are similar to each other and to those obtained for eosin Y. **b** The emission spectra of phloxine after excitation at 530 nm. Excitation at 275 nm results in a fluorescence spectrum similar to that obtained for eosin Y (**c**). **d** The effect of dye concentration on the fluorescence emission by phloxine. The concentration effect is less marked for phloxine than for eosin Y. The 1:100 spectrum corresponds to that of 12 mM phloxine solution

Table 1 Summary of the fluorescence properties of eosin and phloxine presented in Figs. 9 and 10. Values in nanometers

Dye	Medium	Excitation wavelengths	Emission peaks
Eosin	Water	490	545
	Water	265	325, 375, 550
	Ethanol	490	538
Phloxine	Water	530	570
	Water	275	325, 370, 560
	Ethanol	530	560

sorbed. The concentration effect observed is less pronounced for phloxine, probably because it is a less absorbing dye or lighter, according to Lillie (1977).

Since elastic fibers are usually, and perhaps always, faintly stained by eosin, fluorescence microscopy analyses accordingly reveal the distribution of elastic fibers in H&E preparations. Elastic fibers appear highly fluorescent against a completely dark or slightly fluorescent background.

The fluorescence of double-stained sections is, however, highly dependent on the very low staining of elastic fibers under diachromic staining and, additionally, on the staining balance amongst the surrounding structures. The higher the eosin staining the lower the fluorescence emission. Bradbeer et al. (1994) associated the elastic fiber and mineralized bone fluorescence to the lack of staining by azure II and methylene blue after Giemsa staining. We must add that the lack of hematoxylin binding after H&E staining is important but not the only factor involved in the fluorescence of elastic fibers in these preparations since intense staining by eosin/phloxine also abolishes fluorescence as a result of a concentration quenching, as discussed above.

Confocal analyses further improved elastic fibers imaging, resulting in clear-cut pictures of elastic fiber structure and distribution. The possibility of optical section reconstruction with the confocal facility opens new possibilities for applying the procedure to the three-di-

mensional fiber distribution and lamellar structure in elastin-rich tissues during development, aging, and pathophysiological conditions. Indeed, we were able to distinguish aspects of elastic fiber structure and distribution in some skin regions and in several pathological modifications (Carvalho et al. 1996) and in different elastic cartilage (Carvalho and Taboga 1996).

Even though the identification of elastic fibers is made possible by several histochemical methods, including some fluorescence techniques (Vidal 1978, 1980) and immunocytochemistry, we believe that the main contribution of this study is founded on the possibility of examining file material in retrospective investigations without further sectioning and staining. The wide use of H&E in diagnosis and research means that the H&E plus fluorescence microscopy technique is an easy method for the study of elastic fibers.

Though the analysis with the present procedure may be conclusive for the identification of elastic fibers in most cases, it must be kept in mind that the staining behavior of the tissue as a whole is involved with the selective imaging of elastic fiber and that, in certain highly fluorescent H&E stained tissues, the presence of elastic fibers may be obliterated.

Acknowledgements The authors express their gratitude to Nadja Cristina Souza Pinto for the operation of the fluorescence spectrophotometer, to Dr. Narcisa Cunha e Silva and Maurício da Fonseca Vieira (UFRJ, Rio de Janeiro) for free access to the confocal laser scanning equipment and for help with the microscope operation, respectively. H.F.C. thanks Mr. E.J. Silva for continued encouragement and advice. The fluorescence microscope utilized in this work was acquired with funding by FAPESP (Proc.94/5847–9).

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