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Fluorescence Microscopy and Microspectrofluorimetry of Malignant Melanomas, Naevi and Normal Melanocytes

F. W. D. Rost and Julia M. Polak

Department of Histochemistry, Royal Postgraduate Medical School, London W. 12, England

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Fluorescenzmikroskopie und Mikrospektrofluorimetrie der malignen Melanome, Naevi und normale Melanocyten

Zusammenfassung. Die Zellen maligner Melanome, Grenznaevuszellen und normale Melanocyten zeigen in gefrier-getrockneten Gewebsschnitten eine durch Formaldehyd hervorgerufene Fluorescenz. Dieselbe Fluorescenz konnte dann nachgewiesen werden, wenn die Gewebsstücke normal Formol-fixiert und in Paraffin eingebettet waren. Mikrospektrofluorimetrische Untersuchungen zeigten, daß der Charakter der Fluorescenz in allen drei Zelltypen ähnlich ist mit einem Aktivierungs- und Emissionsmaximum bei ungefähr 440 und 490 nm. Durch Salzsäurebehandlung wurde das Aktivierungsmaximum nicht beeinflußt. Die fluorescenzmikroskopische Untersuchung formaldehydfixierter Gewebsschnitte wird als einfache, wertvolle diagnostische Methode vorgeschlagen.

Summary. The presence of formaldehyde-induced fluorescence in malignant melanoma cells, junctional naevus cells and normal melanocytes has been confirmed in freeze-dried material. It has been found that the same fluorescence can be observed in formalin-fixed "routine" paraffin sections. Microspectrofluorimetry showed that the characteristics of the fluorescence are similar in all 3 types of cells, with excitation and emission maxima at about 440 and 490 nm respectively. The fluorescence excitation maximum is not affected by hydrochloric acid. It is suggested that fluorescence microscopy of formalin fixed sections is a simple and valuable diagnostic method.

Introduction

This paper describes a method for obtaining additional information for the diagnosis of malignant melanomas and melanocyte-containing lesions by fluorescence microscopy of formalin-fixed sections.

Baroni (1933) applied fluorescence microscopy to the study of 3 melanomas of the skin, and reported a yellow fluorescence in the tumour cells and in cells of the basal layer of the epidermis. Control tumours (sarcomas) gave a bluish fluorescence. Unfortunately he did not specify the fixative used, but it was probably formalin. Baroni's work was quoted in Haitinger's textbook (1938, 1959).

Falck *et al.* (1965, 1966a, 1966b) studied malignant melanomas, naevi and normal skin in sections prepared by freeze-drying followed by formaldehyde vapour fixation (Falck *et al.*, 1962), and fluorescence microscopy using a yellow barrier filter. Under these conditions a green to yellow fluorescence was noted in normal melanocytes, in naevus cells showing junctional activity, and in melanoma cells. This fluorescence appeared to be specific to normal and abnormal melanocytes. Ehinger *et al.* (1967) found a similar fluorescence in some but not all of 11 ocular malignant melanomas. Olivecrona and Rorsman (1966b) found an increase in melanocyte fluorescence after Röntgen irradiation. The same authors

(1966a) found no such fluorescence in malignant melanomas in Syrian golden hamsters.

On the basis of biochemical analysis, Falck and his coworkers (1966) concluded that the fluorescence was due at least in part to the presence of dihydroxyphenylalanine (DOPA). In addition, a second substance with a fluorescence emission at about 500 nm was found (Falck *et al.*, 1966c). This second fluorophore was stable to hydrochloric acid: its composition was unknown. Ehinger *et al.* (1969) performed microspectrofluorimetry and found that the fluorescence did not appear to be typical of any catechol substance hitherto investigated.

The present work has been directed in two different channels: first, to the development of a technique for routine use in diagnostic pathology, and secondly, to the investigation of the fluorescence by microspectrofluorimetry.

Materials and Methods

Specimens were obtained at operation, of 5 malignant melanomas (2 of them amelanotic), 2 benign melanotic tumours (1 intradermal naevus and 1 compound naevus), 3 non-melanotic tumours (2 basal cell papillomata and 1 basal cell carcinoma) and 4 pieces of normal skin (2 white and 2 coloured).

Part of each specimen was freeze-dried, fixed in formaldehyde vapour at 80° C for 4 hours, and embedded in paraffin wax. Another part was frozen and sections were cut in a cryostat. In both cases, the methods were as described by Pearse (1968) and by Bancroft (1967). A further part of each lesion was processed by the "routine" paraffin method, including initial fixation in 15% formol-saline and a second formalin fixation bath containing 0.7% mercuric chloride. 3 melanomas and 3 naevi processed by the latter method during the past 7 years were also examined.

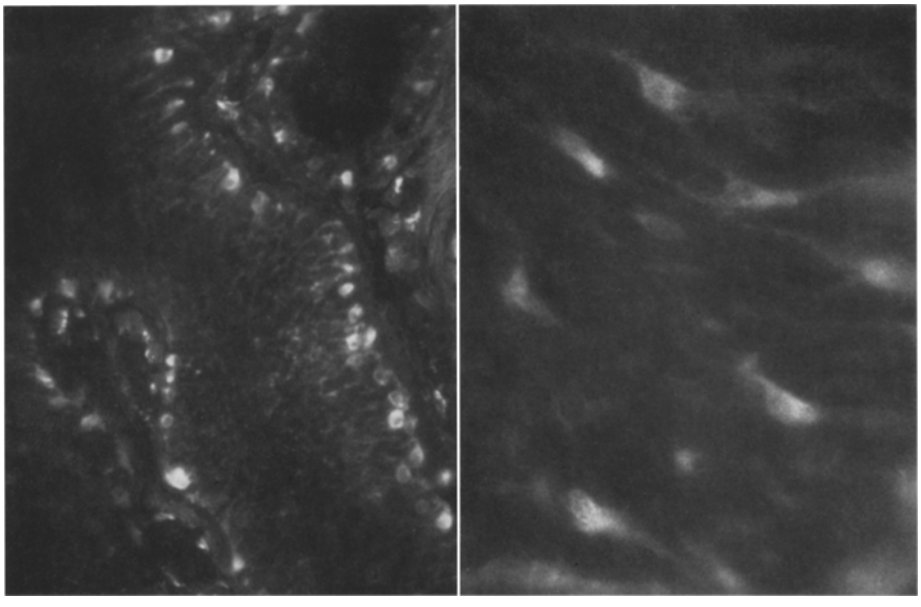
Cryostat sections were stained for melanin by the methods of Schmorl (Bancroft, 1967), and Rio Hortega-Polak (Polak, 1966), and also used for the demonstration of DOPA oxidase (Bancroft, 1967). Sections prepared by both paraffin methods were also stained with haematoxylin and eosin. Models for microspectrofluorimetry were prepared using DOPA in concentrations of $1:10^4$ and $1:10^5$ in 0.1% bovine serum albumen and also in 0.1% polyvinylpyrolidone (PVP); after drying in air the models were exposed to formaldehyde vapuor by the same method as was used for the tissue specimens.

Fluorescence microscopy was carried out with a Leitz Orthoplan microscope, using a mercury arc lamp (HBO 200), BG12 and BG38 excitation filters, and a K530 barrier filter (50% transmission at about 530 nm), and also with a Zeiss (Oberkochen) standard Universal microscope equipped with a similar lamp and filters. The photographs here reproduced were taken on Ilford FP4 film. Microspectrofluorimetry of fluorescent preparations was carried out with a Leitz microspectrograph (Ruch, 1960) modified for fluorescence (Rost and Pearse, 1968; Pearse and Rost, 1969). Epi-illumination (through the objective) was used in all cases: the advantages of this have been discussed by Rigler (1966). In most cases, measurements were made without dewaxing, the section being mounted in paraffin oil. Some were dewaxed in xylene and mounted in paraffin oil. Where possible, both excitation and emission spectra were measured from the same area. There was little fading during irradiation. In all cases, a control blank area was measured and the readings subtracted from the test readings.

The excitation spectra presented (Figs. 3—6) are corrected for the spectral chracteristics of the light source, using a quartz beamsplitter and a Rhodamine B quantum converter (Ritzén, 1967) having a 2-cm. light path for absorption. Corrections have been applied for changing reflectivity of the quartz beamsplitter at different wavelengths. Emission spectra are corrected for the spectral sensitivity of the photomultiplier and for varying bandwidth of the second monochromator: results are expressed in relative quanta per unit quantum energy interval, in partial accordance with the recommendations of Chapman *et al.* (1963).

Results

Our findings in freeze-dried, formalin-vapour fixed (FDFV) material were similar to those of Falck and his colleagues. We were able to confirm the enhanced fluorescence in apparently normal melanocytes in the basal layer of skin over a naevus, and a gradient of fluorescence within the naevus from superficial, brighter layers to deeper, dimmer layers (FALCK *et al.*, 1965). In our series, the only melanoma which did not display fluorescence was a very heavily pigmented one. All the amelanotic melanomas showed fluorescence. There was considerable variation in intensity from cell to cell and from tumour to tumour.

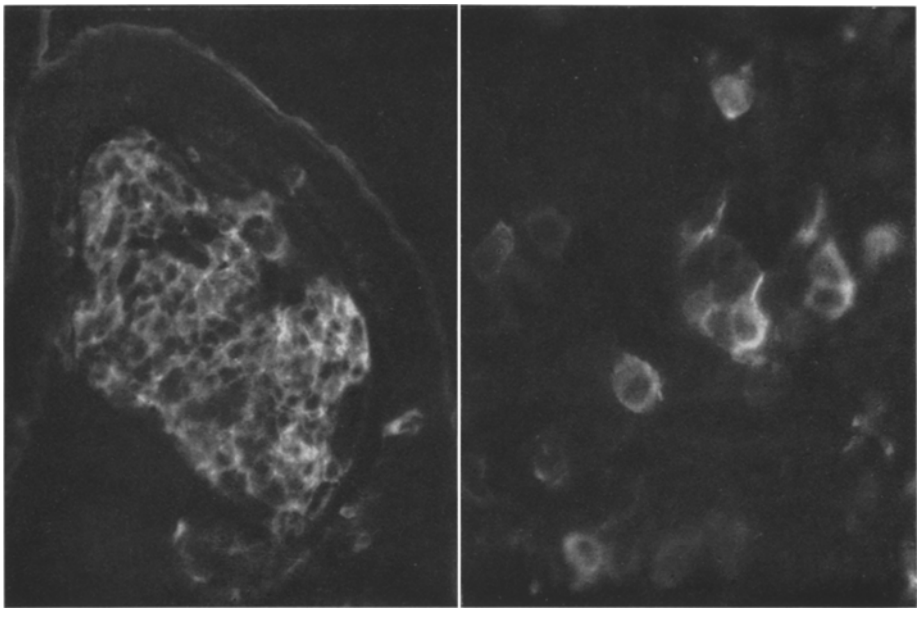


a b

Fig. 1a and b. Benign papilloma showing formaldehyde-induced fluorescence of melanocytes. Freeze-dried tissue fixed in formaldehyde vapour. a Melanocytes in basal layer, ×230. b Dendritic melanocytes in the deeper layers of the tumour, ×560

The FDFV method is not yet universally available in routine pathology laboratories, therefore it seemed desirable to try the original method of ERÄNKÖ (1952), using aqueous formaldehyde and conventional paraffin processing. "Routine" sections proved almost as satisfactory as FDFV sections, both for visual examination and for microspectrofluorimetry, and examination of old sections showed that the fluorescence was stable after more than one year's storage.

Microspectrofluorimetry of normal melanocytes, naevus cells and melanoma cells produced, in most cases, unimodal excitation and emission curves with peaks at about 440 and 490 nm respectively (Figs. 3—5). In one section, bimodal curves with peaks at about 400/440 and 490/520 nm were observed. In some cells, we obtained an emission peak at about 520 nm, but this was changed to 490 nm after dewaxing in xylene. The fluorescence excitation curves were not



a b

Fig. 2. a Compound naevus, showing formaldehyde-induced fluorescence of junctional naevus cells and of melanocytes in overlying basal layer. Freeze-dried, ×280. b Amelanotic malignant melanoma, routine formalin-fixed paraffin section, ×470

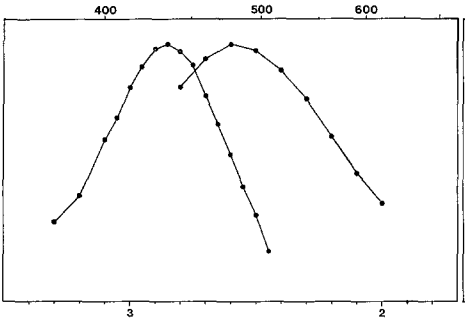


Fig. 3

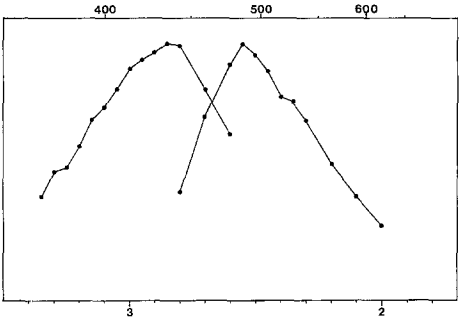


Fig. 4

Fig. 3. Excitation (left) and emission spectra of formaldehyde-induced fluorescence of malignant melanoma cell. Abcissa: quantum energy in eV (lower scale), wavelength in nm (upper scale). Ordinates: Relative quanta emitted per quanta of excitation (excitation spectra) or relative quanta per unit quantum energy interval (emission spectra), arbitrary units

Fig. 4. Excitation and emission spectra of formaldehyde-induced fluorescence of junctional naevus cell. Same scales as Fig. 3

altered by exposure to hydrochloric acid vapour by the method of Björklund *et al.* (1968). In contrast, the DOPA model (Fig. 6) gave fluorescence excitation and emission maxima at about 420 and 490 nm respectively, and the excitation maximum was shifted to 390 nm after a short exposure to hydrochloric acid.

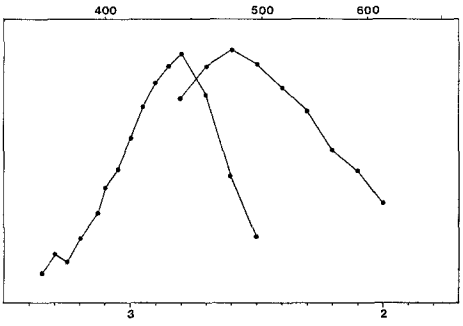


Fig. 5

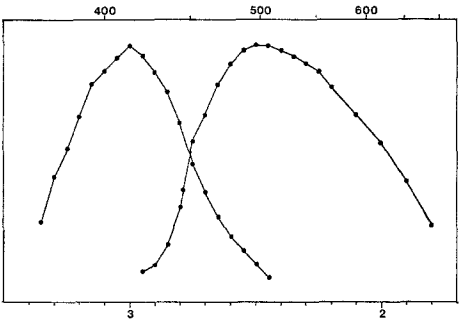


Fig. 6

Fig. 5. Excitation and emission spectra of formaldehyde-induced fluorescence of melanocyte overlying benign naevus. Same scales as Fig. 3

Fig. 6. Excitation and emission spectra of formaldehyde-induced fluorescence of DOPA model. Same scales as Fig. 3

Discussion

We have confirmed the finding of FIF in normal melanocytes, in naevus cells having junctional activity, and in malignant melanoma cells. In melanomas, the intensity of the fluorescence is variable and one might expect occasionally to find a non-fluorescent amelanotic melanoma, although this is probably quite uncommon in view of the combined findings of Falck and his colleagues and of ourselves. The microspectrofluorimetric evidence suggests that the fluorescence is due to a specific substance or group of substances. DOPA, although probably responsible for part of the fluorescence, does not appear to be the main substance responsible, since spectra from the DOPA models are not consistent with those from melanocytes.

There is at present a lack of specific histological methods for diagnosis of malignant melanoma. The most specific method available at present is the DOPA reaction, which requires cryostat sections. The Schmorl test will confirm the presence of melanin, if there is any, and may give a positive result in the absence of obvious melanin: however this test is a non-specific one, since it demonstrates only the property of ferricyanide reduction. Provided incubation times are short (less than 2 min) this is practically restricted to melanin and its precursors. The present method demonstrates a specific property of melanocyte-type cells, and can be applied to routine sections as well as FDFV material. It should therefore prove of easy application and considerable value in the diagnosis of malignant melanomas and naevi.

The method which we recommend for routine use is as follows:

(1) Tissue is fixed in formol-saline (15% formalin recommended).

(2) Tissue is processed to paraffin in the conventional manner.

(3) Unstained sections are dewaxed in xylene and mounted in liquid paraffin or DPX.

(4) Sections are examined by fluorescence microscopy, using blue-violet excitation (e.g. mercury arc lamp with BG12 and BG38 filters) and a yellow barrier filter with cut-off at about 530 nm (e.g. Leitz K530 or Zeiss 51/44).

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Dr. F. W. D. ROST
Department of Histochemistry
Royal Postgraduate Medical School
London, W. 12/England