Early diagnosis of melanotic melanoma based on laser-induced melanin fluorescence

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Abstract. Because of the increasing incidence of skin cancer, interest in using the autofluorescence of skin tissue as a noninvasive tool for early diagnosis is enforced. Focus is especially on malignant melanotic melanoma. On the basis of a newly developed method to selectively excite melanin fluorescence of skin tissue by stepwise two-photon excitation with nanosecond laser pulses at 810 nm, we have investigated information from this melanin fluorescence with respect to the differentiation of pigmented lesions. A distinct difference in the melanin fluorescence spectrum of malignant melanoma (including melanoma *in situ*) when compared to that of benign melanocytic lesions (i.e., common nevi) has been found for freshly excised samples as well as for histopathological samples. There is also specific fluorescence from dysplastic nevi. In this way, early detection of malignant melanoma is possible.
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1 Introduction

Until now fluorescence diagnostics based on the emission of endogenous fluorophores have hardly played a role in the differential diagnosis of skin cancer. Neither conventional (i.e., one-photon fluorescence excitation) nor nonlinear (i.e., multiphoton excitation) has been used. The main reasons are as follows:

1. The autofluorescence of skin tissue usually contains contributions from several fluorophores; the selective excitation of only one type of fluorophore is possible if at all only under extreme conditions (e.g., with subcellular spatial resolution using fs pulses of high flux density,[1] or with dangerous UV-B excitation, e.g., of tryptophane), which are unacceptable for routine *in vivo* diagnostics.

2. Malignant degenerations connected with metabolic changes result at most in changes of the fluorescence quantum yield of certain fluorophores (e.g., NADH/NAD^+) rather than in spectral shifts.[2] This results in changes of the intensity of fluorescence only, which can hardly be quantified absolutely *in vivo*, and obviously, they are no safe basis for a diagnostic method, as is underlined by the unsuccessful activities in this field over decades.[3]

3. Especially melanin, the fluorescence of which (due to the so-called two-edged sword function of melanin[4–6] of being either beneficial or deleterious to the organism) can be expected to be a carrier of information, with respect to malignant degenerations of pigmented skin lesions, has an extremely low-fluorescence quantum yield. For the main melanin types, eumelanin[7] and pheomelanin,[8] it is on the order of 10^{-4}. This, in addition to the problems described above, has

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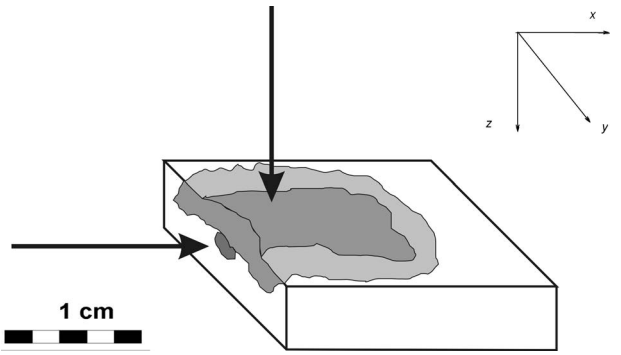


Fig. 1 Schematic drawing of a paraffin slide from a histopathological preparation of a pigmented lesion. The two arrows indicate the different possibilities for fluorescence excitation; vertical array (z direction): excitation perpendicular to the skin surface, horizontal array (x direction): excitation parallel to the skin surface. The sample holder in the measuring device (LIMES 16-P) allows variation of excitation (scanning) in all three directions x, y, z (see text).

until recently been a strict barrier to selectively obtain the melanin fluorescence of skin tissue.

Lately, the decisive progress to optically prepare the practically pure melanin fluorescence from mixtures of several chromatophores (such as, e.g., in skin tissue *in vivo* or in histopathological preparations, in the retinal pigment epithelium, and also in human hairs) has become possible by the rather unusual mode of *nanosecond* laser pulse-based *stepwise* two-photon excitation.[9,10] The crucial points of this technique are briefly explained in Sec. 2. Having, for the first time the melanin fluorescence separated from any other autofluorescence, we present the first results in this paper in relation to the central question: What is the content of information of the melanin fluorescence from tissue with respect to malignant melanotic degeneration?

2 Materials and Methods

2.1 *Samples of Human Skin Tissue*

Twenty-seven histopathological samples of pigmented melanocytic lesions (benign nevi as well as malignant melanomas), formalin fixed, embedded in paraffin, and stained according to general proceedings without any dyeing, have been investigated. Among the several nevus types, there were eight compound nevi, six junctional nevi, two dermal nevi, and two compound nevi with criteria of atypia (nevus Spitz). Furthermore we investigated nine malignant melanomas, including three melanomas *in situ*, one superficial spreading malignant melanoma, and five nodular malignant melanomas. The histological slides were cut vertical (y-z in Fig. 1) perpendicular to the skin surface (y-z in Fig. 1). Thin sections down to $y=5$ μm could be measured. The fluorescence spectra were excited *parallel* to the skin surface (x direction) in a definite, variable depth (z direction, see Fig. 1).

Among some of these samples, it was possible to perform fluorescence excitation *perpendicular* to the skin surface (z direction) (see also Fig. 1). Afterward, this mode of excitation was applied *ex vivo* to 150 freshly excised samples of melanocytic pigmented lesions, including—according to the subsequent histological findings—also the broad spectrum from common melanocytic nevi to malignant melanoma, as described above.

2.2 *Principles of a Fluorescence Measuring Device, Selectively Adapted to Melanin*

The scheme of the fluorescence measuring device LIMES 16-P (LTB Lasertechnik Berlin, Berlin, Germany) used is shown in Fig. 2. Excitation is realized by nitrogen laser-pumped dye laser pulses with a center wavelength at 810 nm and 2.5-ns duration, repetition rate 30 Hz, pulse energy 5 μJ (Dye Laser, LTB Lasertechnik Berlin, Berlin, Germany). The optical setup is based on an inverted microscope (Rapp Opto-Electronic Hamburg, Germany); the laser beam is directed by a dielectric mirror (Beamsplitter DCSP 725, AHF Analysetechnik AG Tübingen) to the long-distance objective L1 (10× PlanC N, NA=0.25, Olympus) and focused to a spot of 50 μm diam. The two-photon excited fluorescence is collected by the same objective, passes through the dielectric mirror and a bandpass filter to attenuate the reflected infrared light of the laser, and is focused by a lens L2 into a 400-μm fiber. The dispersion of the Spectrometer (CP140-1602, Horiba Jobin Yvon) and the 16-channel photomultiplier (R5900U-03-L16, Hamamatsu Photonics) with a channel width of 1 mm result in a spectral resolution of 17 nm per

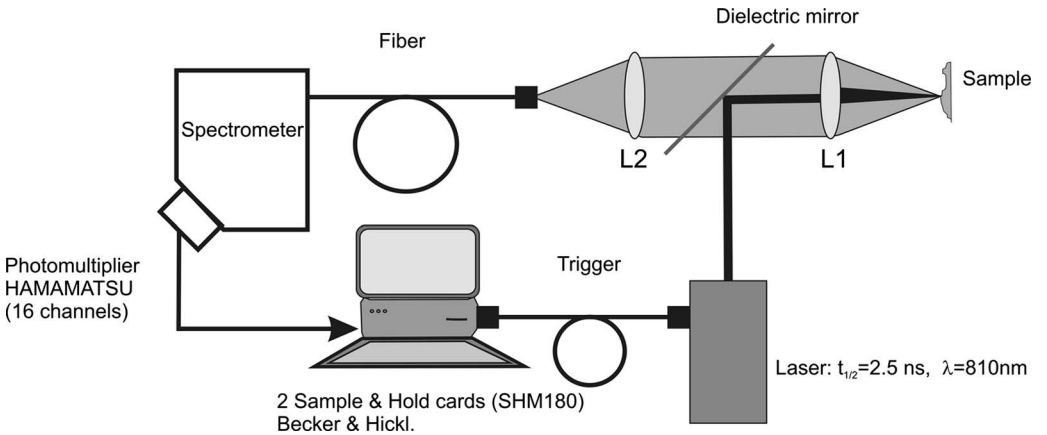


Fig. 2 Scheme of the fluorescence measuring device LIMES 16-P (for details, see text).

channel. The 16 signals are measured with two sample and hold cards (SHM180, Becker & Hickl, Berlin, Germany) that are triggered electronically with a trigger generator (LTB Lasertechnik Berlin, Berlin, Germany) simultaneously with the laser.

3 Experimental Details

The duration of measurement per circular tissue area of 50 μm diam is 10 s, resulting from sampling over 300 measurements. There is a possibility of scanning over the sample region of interest. The sample is fixed on a cover slip that can be moved with an *xyz* stage (r-d-s motion, Strausberg, Germany). The region of interest is selected prior to the spectral fluorescence measurements by a fluorescence imaging procedure based on 337-nm one-photon excitation of the whole sample. In the case of fluorescence excitation perpendicular to the skin surface (with *ex vivo* samples and specially prepared histological preparations), z variation allows an in-depth change of the excited volume through the epidermis and basal layer up to at least 500 μm in the dermis.

In this respect, it is worthy to note that simultaneously with the two-photon excited fluorescence a signal at 405 nm occurs as soon as the probed volume contains (ordered) collagen. This is the second harmonic generation (SHG) of the exciting radiation. This SHG signal is useful information to mark the epidermis-dermis border. Moreover, variations in the ratio of SHG to fluorescence can be used as indicator of laser-induced thermal damage.[11]

Both with histopathological samples and with freshly excised samples, reproducibility of the fluorescence spectra has been confirmed (*i*) by repetition of the measurement immediately after finishing a scan and (*ii*) by tenfold repetition at individual areas. In case of simultaneous occurrence of fluorescence and SHG, their intensity ratio has been found highly stable also within 10 repetitions of the scan, which gives a hint toward the absence of any photodamage. Moreover, all samples used for the laser-excited fluorescence diagnostics were examined by histopathologic inspections. Until now, there is no photodamage detectable by the histopathologic method.

Possible distortions of the melanin-dominated spectra by exogenous fluorophores, by hemoglobin, by hair shafts, and by locally applied medical substances can be easily identified by their specific fingerprints. Details will be described elsewhere.

4 Physical Background

The laser wavelength of 810 nm allows the realization of *stepwise* two-photon excitation of melanin fluorescence (not that this is a special type of fluorescence originating in a higher excited state[12]). The basis of this stepwise excitation process is a sufficient absorption at 810 nm from the ground state (σ_1) as well as from the first excited state (σ_2). The former is obvious from the well-known conventional absorption spectrum of melanin; the latter follows from respective nonlinear absorption measurements, which show that $\sigma_2 \cong \sigma_1$.[13] With all other endogenous fluorophores of skin tissue, σ_1 at 810 nm is vanishingly small, therefore they emit fluorescence at this excitation wavelength—if at all—only via *simultaneous* two-photon excitation. These different two-photon processes result in a first discrimination in the fluorescence intensities in favor of the stepwise excited melanin, as can be seen from the following analytical expressions for the normalized population densities at pulse maximum of the two-photon excited fluorescence levels:[14]

for simultaneous two-photon absorption:

$$n_{\mathrm{sim}}(I_{\max}) = 0.38\sigma^{(2)}\tau I_{\max}^2, \tag{1}$$

for stepwise two-photon absorption:

$$n_{\mathrm{step}}(I_{\max}) = 0.14\sigma_1\sigma_2\tau^2 I_{\max}^2. \tag{2}$$

Here, $I_{\max}$ is the peak intensity of the laser pulse, τ is the pulse duration [full width at half maximum (FWHM)], $\sigma^{(2)}$ is the two-photon absorption cross section, σ_1 and σ_2 are the one-photon absorption cross sections of the two consecutive absorption steps. From Eqs. (1) and (2), it follows that, under identical short pulse excitation conditions, the population density is distinctly higher in the stepwise populated fluorescence level as compared to that of simultaneous excitation. Assuming, for example, 100-fs pulses in the 800-nm range with fixed $I_{\max}$ and average cross sections for the skin fluorophores [$\sigma_1 \approx \sigma_2 \approx 5 \times 10^{-18}$ cm^2 for melanin; $\sigma^{(2)} \leqslant 10^{-51}$ cm^4 s for flavine adenine dinucleotide (FAD)[15] or reduced nicotinamide adenine dinucleotide (NADH)[15]], the product 0.14 $\sigma_1\sigma_2\tau^2$ results in an at least three orders of magnitude higher population n_{step} as compared to n_{sim}, the latter determined by the product 0.38 $\sigma^{(2)}\tau$. This way melanin can partly compensate its very low fluorescence yield (for further details, see Ref. 14).

The second discrimination is based on the different pulse duration dependence of the two excitation modes; see also Eqs. (1) and (2). As long as the lifetime of the intermediate energy level is in the order of the pulse duration or even longer, prolongation of the pulse duration is very much in favor of fluorescence via stepwise excitation (its intensity scales with the square of pulse duration, melanin) as compared to the simultaneous two-photon excitation [which scales linearly with pulse duration (e.g., FAD, NADH)]. The decay of the melanin fluorescence from the intermediate level (i.e., after one-photon excitation) is multiexponential with one or two components in the nanosecond range, depending on the excitation wavelength and on the solvent.[16] This is the reason for using nanosecond pulses instead of femtosecond pulses, which are usually used in nonlinear spectroscopy.

These two specifics of fluorescence excitation strictly minimize the fluorescence of all endogenous fluorophores, except of melanin, so that the latter can be measured very selectively. For a simple *in vitro* demonstration, the one- and two-photon excited fluorescence of dimethyl sulfoxide (DMSO) solutions of FAD and melanin is shown [Fig. 3(a)], as well as that of a mixture of both [Fig. 3(b)]. Whereas with 405-nm excitation, FAD clearly dominates the fluorescence of the mixture, two-photon excitation at 810 nm of the mixture shows the opposite: practically pure melanin fluorescence. (With respect to the spectral shapes of melanin-dominated fluorescence from tissues shown below, it should be mentioned that melanin fluorescence is strongly dependent on the microenvironment and the configuration, e.g., for solvatochromism; see Ref. 14).

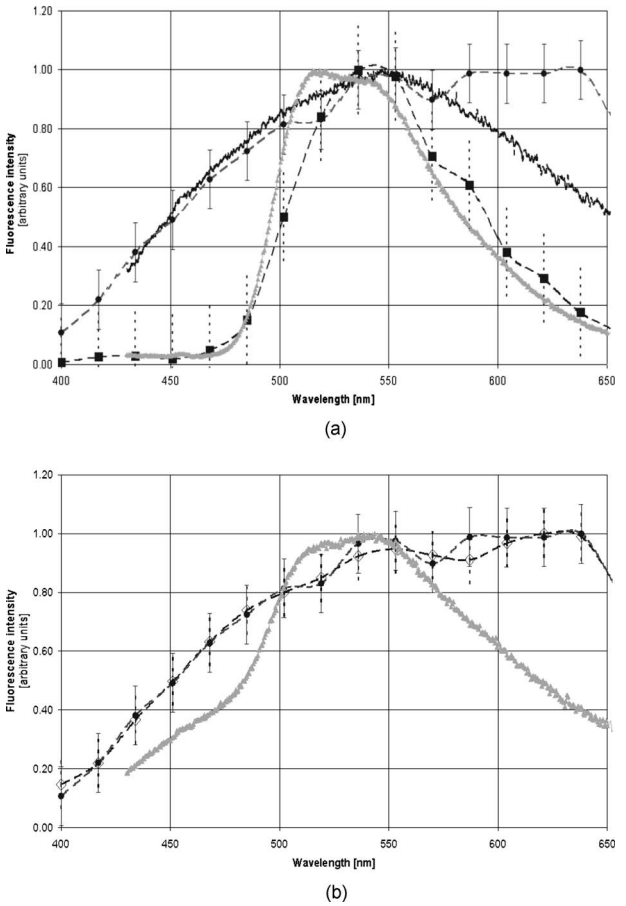


Fig. 3 (a) Fluorescence spectra of oxidized FAD, dissolved in DMSO and excited at 405 nm (continuous line with triangles), as well as excited at 810 nm (black squares, with error bars); and fluorescence spectra of melanin, dissolved in DMSO and excited at 405 nm (continuous line), as well as excited at 810 nm (black dots, with error bars). (b) Fluorescence spectra of a mixture of FAD and melanin, dissolved in DMSO and excited at 405 nm (continuous line with triangles), as well as excited at 810 nm (rhombuses, with error bars). For comparison, a repetition of the 810 nm excited fluorescence of pure melanin/DMSO from figure (a) (black dots, with error bars) is also shown. Measuring conditions for (a) and (b): sample thickness 1 mm; o.d. at 405 nm of the pure FAD solution: 0.75 cm^{-1}, o.d. at 405 nm of the pure melanin solution: 2.23 cm^{-1}. The FAD/melanin-mixture (b) is a 1:50 volume composition of the pure FAD and melanin solutions used for the measurements shown in (a). The 405-nm excited fluorescence spectra were measured with a Fluorolog FL-112 (Jobin-Yvon, Longjumeau, France). The 810-nm excited fluorescence spectra were obtained with the LIMES 16-P, like all other fluorescence spectra shown in Figs. 5 and 6.

Regardless of how selective the melanin fluorescence of skin tissue can be excited, it is ultraweak and needs a sensitive detection system, which is realized on the basis of a 16-channel photomultiplier (Fig. 2).

5 Results and Discussion

A characteristic example of a melanin-dominated fluorescence spectrum from a common nevus in a usual histopathological slide (formalin fixed, paraffin embedded), excited in the above-described manner (810 nm, 2.5 ns) parallel to the skin surface (see Fig. 1), is shown in Fig. 4(a). A fluorescence band, peaking in the blue spectral range at ~480 nm, is dominating. This line is a least-squares fit with a sixth-order polynomial of the data points (the squares in Fig. 4), which are root-mean squares from all spectra scanned over the nevus region. The latter is determind by a preceding imaging procedure (described earlier). Here especially, the sample is a junctional nevus, but it is important to note that with all the 16 nevi, which according to the histopathological findings have no indication of dysplasia, fluorescence spectra have been obtained that are identical or very similar to that shown in Fig. 4(a). Their maxima always occur within the 490 ± 15 nm range, and their FWHM fit into 120 ± 30 nm. As a preliminary result, it is worth mentioning that the spectral shape of the fluorescence from the epidermis of normal pigmented regular skin tissue (also formalin fixed, paraffin embedded) also fits into this spectral range of the fluorescence from common nevi of Fig. 3(a). Thus, it seems that fluorescence from melanin, produced in melanocytes, is very similar if not identical to that from melanin produced in nevus cells.

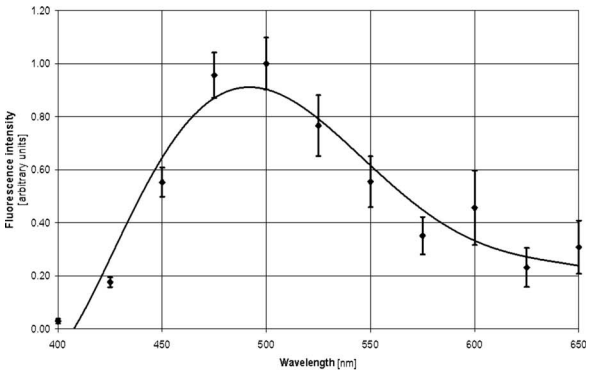
In Fig. 4(b), the solid line, a characteristic example of a fluorescence spectrum from a malignant melanotic melanoma, is shown (nodular malignant melanoma of Clark level V, formalin fixed, paraffin embedded, excited with 810-nm/2.5-ns pulses parallel to the skin surface). This fluorescence is characterized by a dominating band peaking at ~600 nm. The solid line is a least-squares fit with a sixth-order polynomial of the data points, which are root-mean squares from all spectra scanned over the melanoma region.

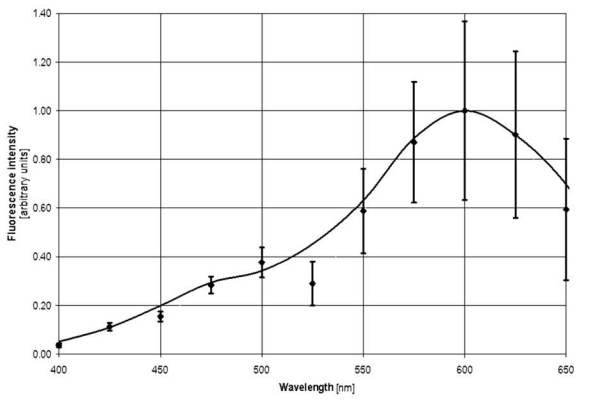
The same or nearly the same fluorescence behavior has been observed for all investigated (scanned) areas in the melanotic melanoma tissue of all nine respective samples listed above. Their maxima always occur within the 600 ± 15 nm range, and their half width at half of maximum (HWHM) on the short-wavelength side of the band fit into 70 ± 10 nm. (The red-most flank of the fluorescence band of Fig. 4(b) is outside of the spectral measuring range). Therefore, it can be stated that the occurrence of this 600-nm band is a fingerprint of malignant melanotic degeneration of tissue in the investigated area.

It is important to note that the shape and maximum of the red fluorescence band do not depend on the type of tumor growth and are the same for all different types of melanomas. Both the fluorescence bands shown in Figs. 4(a) and 4(b) may belong (*i*) to different types of melanin pigments (eumelanin, pheomelanin) or (*ii*) to melanin heteropolymers with a different ratio of these types. There are hints in the literature to the specific role of pheomelanin in dysplastic nevi and in malignant melanotic degeneration.[17,18] Also (*iii*) different stages of melanin aggregation may partly contribute to the 100 nm spectral difference between the fluorescence of common nevi and of malignant melanoma.

These hypotheses are currently under further investigation. It should be mentioned that, especially with respect to its exact molecular structure and its excited state properties, melanin only slowly loses the status of a "black box" (for recent respective reviews, see Ref. 19).

In the case of nevi with histopathologically characterized indications of dysplastic degeneration, there are measuring areas for which the fluorescence spectrum looks like that shown in Fig. 4(c) [i.e., like an intermediate between the be-





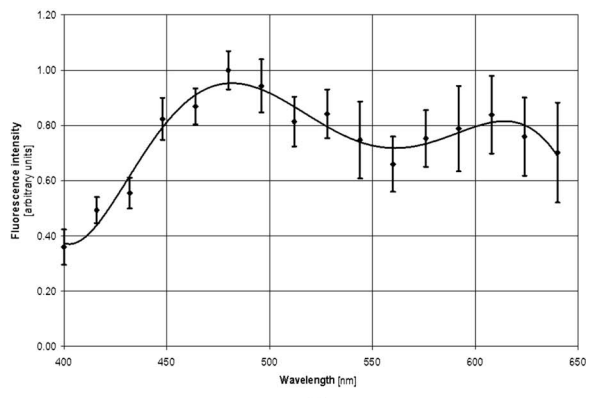
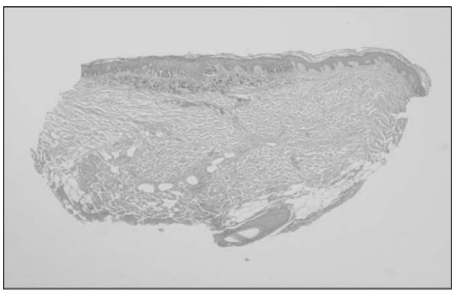
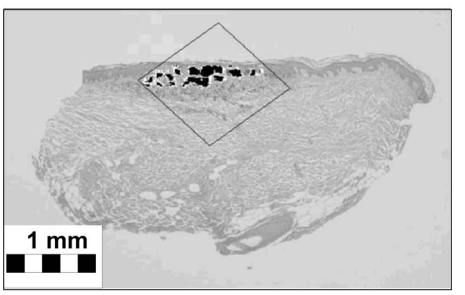


Fig. 4 (a) Characteristic example of the (melanin-dominated) fluorescence spectrum from a junctional nevus in a usual histopathological slide (formalinfixed, paraffinembedded), two-photon excited with 810-nm/2.5-ns pulses parallel to the skin surface. The data points (♦) are root-mean squares from all spectra scanned over the nevus region, the error bars indicate the standard deviation. The solid line is a least-squares fit with sixth-order polynomial of the data points. (b) Characteristic example of the (melanin-dominated) fluorescence spectrum from a nodular malignant melanoma of Clark level V in a usual histopathological slide (formalin fixed, paraffin embedded), two-photon excited with 810-nm/2.5-ns pulses parallel to the skin surface. The data points (♦) are root-mean squares from all spectra scanned over the melanoma region; the error bars indicate the standard deviation. The solid line is a least-squares fit with sixth-order polynomial of the data points. (c) Example of intermediate fluorescence behavior of dysplastic melanocytic lesions; here, it belongs to a compound nevus of Clark type. The data points (♦) are root-mean squares from all spectra scanned over the dysplastic region; the error bars indicate the standard deviation. The solid line is a least-squares fit with sixth-order polynomial of the data points.


(a)



(b)

Fig. 5 (a) Picture of a melanoma *in situ* with a preexisting nevus (histopathological slide). (b) Representation of the borders of a raster (0.1 × 0.1 cm; 500 measuring areas) of a fluorescence scan across a part of the melanoma *in situ* shown above. Within this raster, black areas indicate fluorescence behavior like that shown in Fig. 4(b). For clarity of the representation, indication of the areas of fluorescence like that shown in Fig. 4(c) have been omitted. They surround the black areas shown.

havior shown in Figs. 4(a) and 4(b)]. In these intermediate spectra, the weights of the two bands may vary between the two borders characterized by Figs. 4(a) and 4(b). These 50-μm-diam areas with indication of beginning malignant degeneration may be well separated from each other or they may be connected in varying extent. Our experience thus far suggests a correlation between the percentage of areas with intermediate fluorescence behavior (and their partial connectivity) in the scan region and the degree of dysplasia seen by the dermatopathologist.

In Fig. 5, the fluorescence pattern is shown for a melanoma *in situ*. (a) A microscopic image of a melanoma *in situ* with a preexisting nevus is shown. (b) The borders of a raster of a fluorescence scan across a part of the sample are shown. The extended black regions within this raster represent areas with fluorescence behavior according to Fig. 4(b) (malignant melanotic degeneration). They coincide with the suspect region characterized by the histopathological inspection.

The fluorescence spectra of the histopathological samples shown in Fig. 4 were excited *parallel* to the skin surface (see Fig. 1). In this manner, for example, the vertical extension of malignant melanoma can be determined (with 50-μm resolution). In suitable histopathologic preparations, the fluorescence can be excited also *perpendicular* to the skin surface (Fig. 1). Of course, a spectrum measured in this way samples fluorescence from different skin layers in depth; nevertheless, the characteristic features as indicated in Figs. 4(a) and 4(b) remain. As an example, in Fig. 6 a representative melanin-dominated fluorescence spectrum from a nodular malignant

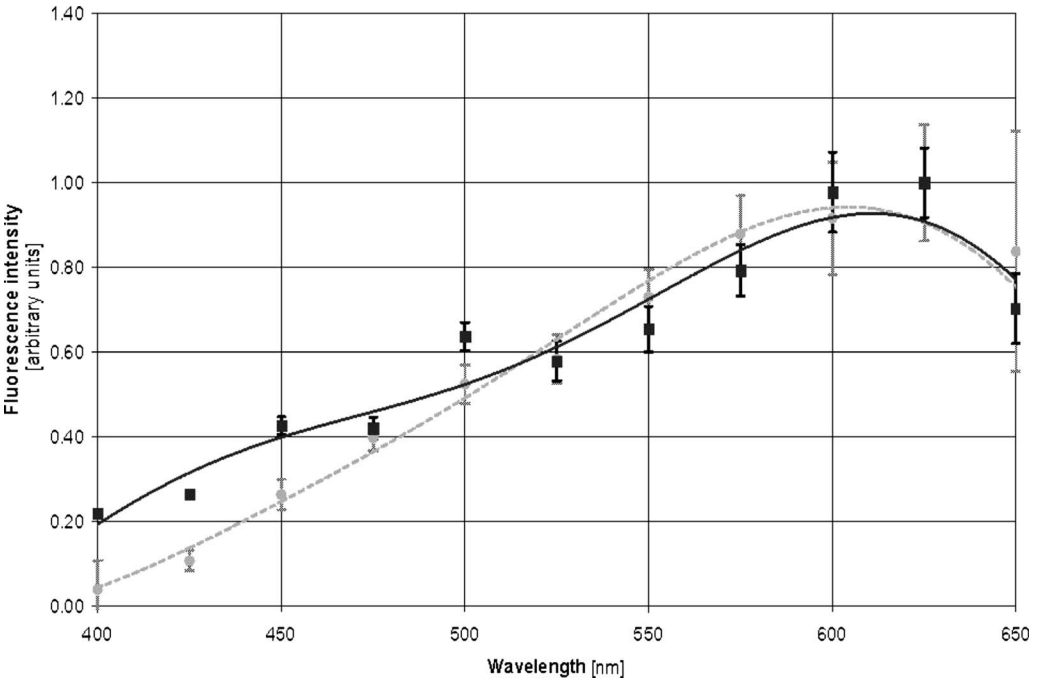


Fig. 6 ● Characteristic example of fluorescence spectrum from a nodular malignant melanoma (histopathological preparation, tumor thickness 4.5 mm, Clark-Level IV), two-photon excited with 810-nm/2.5-ns pulses perpendicular to the skin surface. The broken line is a least-squares fit with sixth-order polynomial of the data points. ■ Characteristic example of fluorescence spectrum from a melanoma *in situ* on a junctional melanocytic nevus (freshly excised sample), two-photon excited with 810-nm/2.5-ns pulses. The continuous line is a least-squares fit with sixth-order polynomial of the data points.

melanoma (tumor thickness 4.5 mm, Clark-level IV, according to the respective histopathological findings), excited perpendicular to the surface, is shown. With this mode of excitation, besides the main aim of the differentiation between common nevus and melanotic degeneration, in the latter case, the horizontal extension of a malignant melanoma can be determined and the walls of the biopsy/excised sample can be characterized with respect to the completeness of tumor excision.

First results with freshly excised skin tissue samples (measured prior to the formalin treatment) indicate a similar fluorescence spectroscopic behavior as the corresponding histopathological samples. In particular, there is also a difference of ~100 nm between the maxima of the "benign" and "malign" fluorescence bands. This holds true even for melanoma *in situ*. An example is also shown in Fig. 6.

6 Conclusion

The possibility of separating the fluorescence of melanin from that of other species in mixtures of fluorophores by combination of a *stepwise* two-photon absorption with *nanosecond*-laser pulse excitation allows, for the first time, a very sensitive approach to the investigation of melanin-based problems (e.g., in dermatology, ophthalmology, and pharmacy). One special example is noninvasive detection of malignant melanoma. From the described measurements of the fluorescence spectra of melanin in histopathological as well as freshly excised samples of pigmented skin lesions, it follows that melanin is a sensitive intrinsic indicator of malignant melanocytic degeneration: The fluorescence spectrum of melanin in common nevi is remarkably different from the fluorescence spectrum of melanin in all types of malignant melanoma (including melanoma *in situ*). Dysplastic nevi show intermediate fluorescence shapes with varying contribution of both "pure" bands shown in Figs. 4(a) and 4(b). This, together with the 50-μm local resolution of the method, allows early detection of malignant melanotic melanoma.

The use of two-photon excited melanin fluorescence as indicator of malignant melanocytic degeneration is a completely new diagnostic approach, different, for example, from all ABCD-rule-based and dermatoscopic methods. The final goal will be *in vivo* fluorescence diagnostics.

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