Selective Femtosecond Pulse-Excitation of Melanin Fluorescence in Tissue

To the Editor:

Until now fluorescence diagnostics have not played a role in the differential diagnosis of melanocytic pigmented lesions, even though a lot of research has been carried out in this area (Sterenborg *et al*, 1994).

Supposition to fluorescence diagnostics of melanoma is either a selective reflection of malignant transformation in the fluorescence properties of the respective tissue (the respective endogenous fluorophore) itself, or a selective enrichment of exogenous fluorophores in melanoma. The problems with the latter have been under careful investigation (Sterenborg *et al*, 1996). In the following, we will refer to the former point, endogenous fluorescence with specific response to malignant transformation. Published data in this respect are scarce and are obviously not well suited as a safe basis of diagnostics.

On the other hand, because of the central role of melanin synthesis as a main phenotype in the pathophysiology in neoplastic transformation of melanocytes (Jimbow *et al*, 1991), and because all melanin response to sunlight (i.e., the processes subsequent to sunlight absorption) is mediated via the fluorescent excited state, important information can be expected from a selective detection of melanin fluorescence from skin tissue (e.g., information about the melanin excited state properties, and, at least implicitly, about the tendency to make useful or deleterious photochemistry). But such selective excitation of melanin emission by conventional one-photon excitation is practically impossible because of overlap with the fluorescence excitation regions of other fluorophores, and because of the extremely low fluorescence quantum yield of melanin.

Here we report on a new approach to get selectively the melanin fluorescence from skin tissue, and we present results on distinct differences of the spectral shape of this fluorescence when originating in excised malignant melanoma, as compared with excised normal healthy tissue as well as common nevus cell nevi.

The basis of the new technique is the unique light absorption property of melanin: it exhibits a monotonously decreasing absorption spectrum between the near UV region and the near IR region. This behavior is the same for melanin in tissue as well as for dissolved synthetic melanin. It is fundamentally different from the absorption of other organic fluorophores, which show nonmonotonously shaped spectra with few absorption bands.

In the red-most tail (NIR) of the melanin absorption it is possible to find spectral regions where there is no overlap with any absorption of another fluorescent tissue component (e.g., around 800 nm). If one excites melanin in such spectral region with femtosecond (fs) laser pulses, fluorescence in the 400–650 nm region occurs (Teuchner *et al*, 1999), which results from consecutive absorption of two NIR photons. This process of stepwise two-photon excitation of melanin fluorescence is different from the generally known process of simultaneous two-photon excitation of fluorescence. The former is a two-step process via a real intermediate excited state energy level, whereas such a real intermediate state is missing in the latter case. Stepwise two-photon excitation needs only two- or more orders of magnitude lower excitation intensity to get the same population density of the fluorescent level as compared with simultaneous excitation.

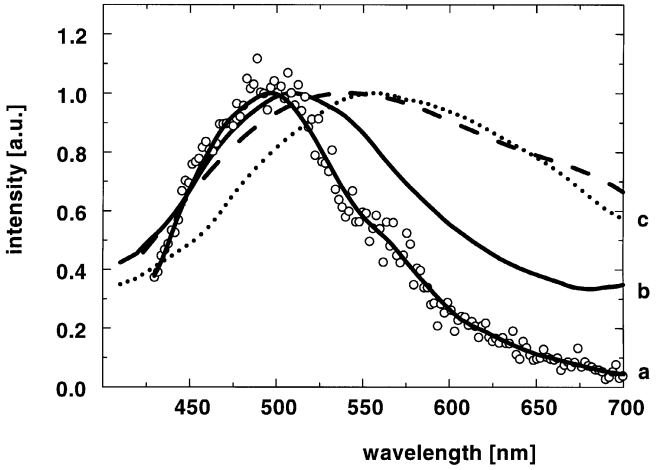

Figure 1. Fluorescence spectra of excised samples of human skin tissue, excited by 120 fs-pulses via 800 nm two-photon absorption. Characteristic examples of (*a*) normal healthy skin tissue, (*b*) common nevus cell nevus, and (*c*) malignant melanoma. ……, nodular malignant melanoma of a 60-y-old woman, Clark Level IV, tumor thickness 1.7 mm according to Breslow; ——, superficial spread malignant melanoma of a 64-y-old woman, Clark Level I. The spectra are normalized; each shape is an average over the experimental data points; in addition, the latter are shown for case (*a*) explicitly.

Experiments with synthetic melanin in different solvents have shown a strong environment-sensitivity of the two-photon excited melanin fluorescence spectrum (Teuchner *et al*, 2000). This has been a basic aid to our hypothesis, that the shape of melanin fluorescence in skin tissue, excited with fs-NIR-pulses, should allow a differentiation between a harmless pigmented skin lesion and malignant melanoma.

In **Fig 1** we present experimental results with intact samples of skin tissue, which support this hypothesis. These samples were excised from patients, prepared as longitudinal sections parallel to the skin surface (area: $\approx 0.5 \times 0.5$ cm^2, thickness: 0.1–0.2 cm) and conserved by keeping them in a NaCl solution (9 g per liter). Together with the NaCl solution they were inserted into standard quartz cuvettes (d = 0.2 cm) for fluorescence studies. The fluorescence spectra were excited with 120 fs/800 nm-pulses (photon flux density: $\approx 10^{28}$ per cm^2 per s,

Manuscript received August 11, 2000; revised November 21, 2000; accepted for publication December 11, 2000.

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repetition rate 1 kHz) and detected by gated photon counting. The experimental method and set-up are described in detail elsewhere (Teuchner *et al*, 2000). Characteristic examples are shown of fluorescence from normal skin tissue (**Fig 1*a***), from a common nevus cell nevus (**Fig 1*b***), as well as from melanoma (**Fig 1*c***). The skin type of all samples was type II–III according to Fitzpatrick. The diagnosis was proven by histology. Until now, we have investigated a total of at least 10 samples each and have found in all cases a characteristic difference between the fluorescence shape of healthy tissue and common nevus cell nevi at the one side and the fluorescence shape of malignant melanoma at the other side, namely in the latter a distinct enhancement of the long-wavelength part connected with a bathochromic shift of the maximum, as is obvious from **Fig 1**. The two examples of fluorescence from malignant melanoma shown in **Fig 1**(***c***) characterize the maximum scatter in the fluorescence shapes of all malignant melanoma samples measured so far. (Conventional 400 nm one-photon excited fluorescence of all samples has also been measured; in this case melanoma do not show a characteristic feature.) We are presently investigating whether the fluorescence behavior according to **Fig 1**(***c***) is unique to malignant melanoma, and whether the increase in the red part of this fluorescence spectrum (as compared with **Fig 1*a***, ***b***) reflects the known increase of the ratio of pheomelanin to eumelanin in the process of malignant transformation (Salopec *et al*, 1991). Preliminary results with few samples of other skin diseases, e.g., basal cell carcinoma, as well as dysplastic nevus cell nevi, are in line with this working hypothesis. The final goal could be a new, noninvasive method of early detection of melanoma.

We gratefully acknowledge the collaboration with the fs-spectroscopy application laboratory of the Max-Born-Institute (Dr. F. Noack). The technical assistance of R. Lendt is appreciated. This work was supported by the Deutsche Forschungsgemeinschaft (grant Le 729/6–1,2).

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