Demarcation of Nonmelanoma Skin Cancer Margins in Thick Excisions Using Multispectral Polarized Light Imaging

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More than a million cases of nonmelanoma skin cancers are diagnosed every year. Treatment of cancer patients could benefit greatly if a real-time, noninvasive, reliable, and cost-effective technique for delineating tumor margins were available. A novel multispectral dye-enhanced polarized light imaging technique that enables rapid imaging of large tumor fields is described. A tunable monochromatic light source and a CCD camera were employed as the imaging device. Linear polarizers were introduced into both the incident and collected light pathways in order to limit the measurement volume to the superficial tissue layers. To enhance the tumor contrast in the image, aqueous solutions of toluidine blue or methylene blue were topically applied to fresh thick skin excisions for several minutes. Then the specimens were rinsed in saline solution. Images were acquired before and after staining at the selected wavelengths. The two sets of wavelengths corresponding to the hemoglobin Soret absorption band and to the absorption bands of the dyes were used to demarcate the areas of enhanced hemoglobin and dye absorption, respectively. The resulting images demonstrate that staining significantly enhances contrast of the tumor in the image and enables reliable delineation of cancer. Locations and shapes of tumor lobules revealed by polarized light images closely correspond to those found in Mohs frozen sections for 41 specimens out of 45. The study demonstrates that the suggested technique has significant potential as a guidance tool in tumor excision surgery. Key words: dyes/multispectral polarized light imaging/nonmelanoma skin cancer/skin. J Invest Dermatol 121:259–266, 2003

Nonmelanoma skin cancers are the most common forms of human cancer. About 75% of all skin cancers are basal cell carcinomas (BCC) and about 20% are squamous cell carcinomas (SCC). These cancers are a major cause of morbidity in the Caucasian population. They commonly appear on sun-exposed areas of the body such as the head and neck. As many tumors occur on the face it is imperative to preserve normal skin surrounding the tumor. Unfortunately, most of these tumors have poorly defined boundaries, which makes visual detection of the tumor borders and, consequently, precise excision a challenging problem. In the USA, Mohs micrographic surgery (MMS) (Mohs, 1941; Mikhail, 1991) is an accepted procedure that removes as little normal skin as possible while providing the highest cure rate. Using detailed mapping and complete microscopic control of the excised lesion the Mohs surgeon can pinpoint areas at the surgical margins involved with cancer that are otherwise invisible to the naked eye. Although precise and accurate, MMS is also a time-consuming and staff-intensive procedure. It requires a surgeon trained in dermatopathology, a dedicated laboratory, and a technician to prepare and evaluate frozen sections. Because of these shortcomings, MMS is used in the minority of cases. Therefore, a simpler, more time-efficient method would be valuable.

In this work, the possibility of using a multispectral dye-enhanced polarized light imaging (PLI) technique as a novel approach to the real-time noninvasive assessment of tumor margins is evaluated. Multispectral imaging is a spectroscopic technique that enables localization and effective discrimination of the chromophores, which absorb in different spectral regions. This technique has been successfully used in different biomedical applications (Dickinson et al., 2001; Cerussi et al., 2002). Multispectral imaging may be particularly valuable for in vivo intraoperative tumor margin demarcation, as blood, which is always present in the surgical bed, makes visual (white light) inspection of the tumor margins difficult. The use of polarized light allows imaging of the superficial tissue layers only. When the light incident on the sample is linearly polarized, subtraction of two images acquired with the co-polarized \( I_\parallel \) and cross-polarized \( I_\perp \) light can be used to largely isolate the single-scattered component, which arises mainly from superficial skin layers (Backman et al., 2000). The advantages of PLI include the ability to image comparatively thin tissue layers (\( \approx 75–200 \mu \text{m} \) in the visible spectral range) and to retain a large field of view. Jacques et al. (2000) recently used white polarized light digital imaging to evaluate pigmented skin lesions. In their work a polarization image, \( I_{\text{pol}} = (I_\parallel - I_\perp)/(I_\parallel + I_\perp) \), was created and analyzed. Melanin strongly scatters light (Vitkin et al., 1994), producing bright areas with excellent contrast in pigmented lesions. Such high contrast based on scattering would not be expected to occur reliably in nonmelanoma skin cancers, which contain variable amounts of melanin. Therefore, it might be beneficial to combine spectrally resolved PLI with tumor staining for enhancing tumor contrast.

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in the images. Application of nontoxic contrast agents is practical during surgery for skin cancer. Contrast agents that alter the imaginary part of the refractive index, the dyes, increase absorption of light by the tissue. The structures that retain a dye appear darker than the surrounding medium. Dyes increase absorption of light of specific wavelengths, thus allowing spectral selectivity. The dyes that are selectively retained by cancerous tissue have been applied previously to aid in visual examination of oral, bladder, and cervix lesions. Phenothiazinium dyes including methylene blue (MB) and toluidine blue (TB) in particular have been used for staining various carcinomas in vivo (Kaisary, 1986; Eisen et al., 1999). In addition, TB is routinely used to stain fresh-frozen tissue sections during MMS (Gross et al., 1999). Phenothiazinium dyes are accumulated to a much greater extent in mitochondria of carcinoma cells compared to normal cells (Oseroff et al., 1986). MB has been successfully applied to grossly demarcate neoplastic tumors in bladder (Fukui et al., 1983; Gill et al., 1984), tumors of pancreas (Fedorak et al., 1993), and Barrett's esophagus metaplasia (Canto et al., 1996). TB has been used topically to detect cervical carcinoma (Richart, 1963), oral carcinoma (Niebel and Chomet, 1965), and Barrett's esophagus metaplasia (Eisen et al., 1999).

In this in vivo study the potential of the suggested multispectral dye-enhanced PLI technique to delineate the margins of nonmalignant skin cancer was investigated. For this purpose a laboratory set-up, which enables multi-wavelength (five wavelengths, including 410 nm, 600 nm, 610 nm, 710 nm, and 710 nm) rapid imaging (within 10 s) of thick tissues and large surfaces (2.8 cm×2.5 cm) was built. The polarized light images of thick skin excisions were acquired before and after staining with TB or MB. The acquired images were processed and superficial images for four wavelengths, including 410 nm, 600 nm, 610 nm, and 620 nm, were obtained and analyzed. The ability of the developed imaging technique to demarcate tumor margins and discriminate endogenous (blood) from exogenous (dye) chromophores was evaluated. Different types of BCC and SCC skin excisions were examined. The superficial images of these lesions were compared with the corresponding Mohs frozen sections processed during Mohs surgery.

**MATERIALS AND METHODS**

**Tissue preparation and handling** Discarded tumor material was received from Mohs micrographic surgeries performed at the Dermatologic Surgery Unit of Massachusetts General Hospital under an IRB-approved protocol. In order to preserve the standard Mohs methodology, we used skin excisions that remained after Mohs histologic analysis and were therefore subject to freezing in microcryotome. After thawing in isotonic Dulbecco's phosphate-buffered solution (DPBS, pH 7.4), the unstained tissue was imaged as described below. Then the tissue was briefly (up to 5 min) stained with 0.01%–0.05% DPBS dye solution. To remove excess of the dye after staining the specimens were rinsed in DPBS. The tissue was imaged again after staining. For imaging, the tissue was placed in a Petri dish on a gauze soaked in saline solution and covered with a coverslip. In total, 45 skin excisions of BCC (including nodular, micronodular, infiltrative, and superficial) and SCC (including invasive SCC and SCC in situ) were obtained from 38 patients.

**Phenothiazinium dyes** Commercially available MB (MB 1% injection, USP: American Regent Laboratories, Aquadilla, Puerto Rico, USA) and TB (TB 1% AQ, LC26656-1, Fischer Scientific Company, Pittsburgh, PA, USA) were used. MB and TB have similar chemical structures (Fig. 1) and exhibit similar physicochemical properties. The blue color of the dyes is caused by the strong absorption band in the 550–700 nm region. In contrast, absorption in skin, which is dominated by two main chromophores, melanin and hemoglobin, exhibits a maximum around 400 nm. Absorption spectra of MB, TB, and human skin are presented in Fig. 2. Figure 2 suggests that spectrally resolved imaging in the range from 400 nm to 700 nm can delineate the areas of enhanced blood absorption and the areas of enhanced dye absorption.

**Imaging equipment** To enable automated multi-wavelength PLI we built a laboratory device schematically presented in Fig. 3. A xenon arc lamp (Lambda LS, Sutter, Novato, CA) combined with interference filters...
was employed as a monochromatic light source and a CCD camera (CoolSNAP Monochrome Photometrics, Roper Scientific, Tucson, AZ) as an imaging device. Linearly polarizing filters were introduced into the pathways of incident light and light collected by the camera. The assembled set-up allowed acquisition and processing of conventional images and polarized light images at the selected wavelengths ($\lambda = 410$ nm, 600 nm, 680 nm, 620 nm, 710 nm) in the visible spectral range. The system provided rapid automatic image acquisition (total acquisition time for all five wavelengths was within 10 s), a large field of view (maximum 2.8 cm $\times$ 2.5 cm), and lateral resolution of approximately 30 $\mu$m.

**Imaging technique** For the detection of skin lesions before and during surgery it is advantageous to acquire superficial images of the lesion. To achieve this goal while retaining a large field of view we employed PLI. PLI enables superficial imaging because single scattering does not change the polarization of the elastically scattered light significantly, whereas polarization of the multiply scattered light is randomized. Therefore, when skin is illuminated with linearly polarized light, and two images are acquired using the remitted light polarized in the directions parallel ($I_1$) and perpendicular ($I_2$) to the polarization of the incident light, the difference image ($I_d = I_1 - I_2$) is produced mainly by single-scattered light. The depth where the first backscattering event occurs is an adequate approximation for the thickness of the tissue layer, which contributes dominantly to the measured signal (imaging depth). This imaging depth $D$ is defined by the optical properties, i.e., the scattering coefficient $\mu_s$ and the anisotropy factor $g$, of the investigated medium and can be expressed as $D = I \mu_s (1 - g)$. Using optical properties of skin from the literature (Svaasand et al., 1995; Douven et al., 2000), it is possible to estimate the dependence of the imaging depth of the skin on the illumination wavelength. This dependence is presented in Fig. 4. It shows that the imaging depth increases with wavelength. At 400 nm the skin section thickness is approximately 75 $\mu$m, at 500 nm approximately 100 $\mu$m, and at 700 nm approximately 200 $\mu$m.

As shown in Fig. 2, the wavelength of 410 nm corresponds to the hemoglobin absorption band, whereas the wavelengths of 600 nm, 680 nm, and 620 nm correspond to the absorption bands of TB and MB. These wavelengths were used to demarcate the areas of enhanced hemoglobin and dye absorption, respectively. The images acquired at the reference wavelength $\lambda_r = 780$ nm were used for the background subtraction as neither hemoglobin nor the dyes absorb the light at this wavelength considerably. The acquired images were processed in the following way. First, to obtain the superficial images, the difference images $I_d$ were calculated for each wavelength. Second, to reject the background signal, the resulting images $I'_d = I'_d - I'_d$, for $\lambda = 410$ nm,

![Figure 4](image1.png)

**Figure 4.** Dependence of the imaging depth (superficial image section thickness) on the wavelength of imaging light estimated using the known optical properties of skin. Single scattering does not change polarization of the elastically scattered light, whereas polarization of the multiply scattered light is randomized. Therefore, when skin is illuminated with linearly polarized light and two images are acquired using the remitted light polarized in the directions parallel ($I_1$) and perpendicular ($I_2$) to the polarization of the incident light, the difference image ($I_d = I_1 - I_2$) is produced mainly by single-scattered light. Thus the depth where the first backscattering event occurs is an adequate approximation for the thickness of the tissue layer, which contributes dominantly to the measured signal (imaging depth). This imaging depth $D$ is defined by the scattering coefficient $\mu_s$ and the anisotropy factor $g$ of the skin: $D = I \mu_s (1 - g)$.

![Figure 5](image2.png)

**Figure 5.** Images of skin with infiltrative BCC (site: lip) acquired at the wavelength $\lambda = 410$ nm. In the conventional images, acquired before (a) and after (b) TB staining, blood appears dark. The tumor is not apparent in the images due to high blood content and absorption (a, b) and negligible absorption of TB (b) at 410 nm. In contrast, the tumor can be clearly delineated as a structureless area in the superficial image, IΔ410, at 410 nm (c, arrow). Bar: 5 mm.
600 nm, 610 nm, and 620 nm, were obtained, analyzed, and compared to histopathology.

**Histopathology** Horizontal sections were prepared by a Mohs histotechnician during surgery in the following way. Tissue removed from patients undergoing nonmelanoma skin cancer treatment was frozen in optimal cutting temperature compound and processed in the standard horizontal sectioning technique of Mohs (Mohs, 1941; Mikhail, 1991). Sections 5 μm thick were transferred to glass slides and stained with hematoxylin–eosin (H&E). These frozen sections were analyzed for residual tumor at the margins. The last frozen section generated during the procedure was then compared to the superficial images of the remaining discarded piece of excision obtained using the technique described above. After imaging the tissue was fixed in 10% formalin and processed for routine H&E-stained formalin-fixed paraffin-embedded permanent histopathology for control. Horizontal sections were prepared in a way similar to the Mohs sectioning technique.

**RESULTS**

**Demarcation of tumor margins and discrimination of the chromophores using multispectral dye-enhanced PLI** The developed imaging technique enabled demarcation of tumor margins and discrimination of endogenous (blood) from exogenous (dye) chromophores. Example images of infiltrative morphea-form BCC obtained at the wavelengths of 410 nm and 610 nm before and after staining are presented in Figs 5 and 6. The images acquired at the wavelength of 410 nm before and after dye application are presented in Fig 5(a) and (b), respectively. In Fig 5(a) and (b) the areas contaminated with blood appear dark in the image. On the upper right and lower left boundaries of the sample the Mohs stain can be seen. Due to high blood content in the sample and enhanced hemoglobin absorption at 410 nm, it is difficult to locate the tumor in the conventional images, presented in Fig 5(a), (b). Figure 5(a) (specimen before staining) does not differ significantly from Fig 5(b) (specimen after staining) because, unlike blood, TB does not absorb light in the blue spectral range. Some blood was partially removed from the skin during staining and rinsing procedures, accounting for the differences in the adherent blood patterns in the images in Fig 5(a), (b). The superficial image $I^H_410$ at 410 nm is shown in Fig 5(c). The section thickness of the superficial image obtained at 410 nm is approximately 75 μm (see Fig 3). The absence of dark areas in the image $I^H_410$ suggests that there is no hemoglobin within the most superficial 75 μm of the tissue. Superficial hemoglobin was removed from the skin during staining and rinsing. In Fig 6(a), (b) we present the conventional images of the same infiltrative BCC tumor acquired before (Fig 6a) and after (Fig 6b) TB staining at the wavelength of 610 nm. This wavelength corresponds to the absorption band of TB (see Fig 2). Figure 6(c) presents a superficial image, $I^H_610$, of the tumor acquired at the same wavelength of 610 nm. In contrast to Fig 5(a), in Fig 6(a) no blood is noticeable, as at 610 nm hemoglobin absorption is weak. Comparison of Fig 6(a) (image of unstained tissue) and Fig 6(b) (image of the stained tissue)
confirms that the dye stains the tumor to a much greater extent than healthy tissue and shows that topical application of TB significantly enhances contrast of the tumor in the image. Section thickness of the superficial image at 610 nm is approximately 150 μm, which is much thicker than a Mohs frozen section (5 μm). Nonetheless, the dark area in the image clearly delineates lesion boundaries, which correlate well with the margins outlined by the surgeon in the image of the histologic slide of the same tumor (Fig 6d). It should be noted that the tumor could be identified in each of the superficial images presented (i.e., $I_{410}^\Delta$ (Fig 5c) and $I_{610}^\Delta$ (Fig 6c)). In the images $I_{410}^\Delta$ acquired at 410 nm, the tumor appears as a structureless, homogeneous area, whereas in the image $I_{610}^\Delta$ the contrast of the tumor in comparison to normal skin is enhanced by the increased absorption of the dye that is retained in the tumor.

Comparison of superficial, dye-enhanced conventional, and dye-enhanced superficial images with histopathology

Example images of the nodular and micronodular BCC acquired at a wavelength of 620 nm are shown in Fig 7. Conventional and superficial images of unstained tissue are

![Figure 7](image_url)

**Figure 7.** Images of skin with nodular and micronodular BCC (site: nose) acquired at the wavelength $\lambda = 620$ nm. Tumor margins are difficult to identify in the conventional image of the tissue acquired before dye application (a). In the superficial image (b) the tumor boundaries could be delineated even without staining (b, arrow). In the conventional (c) and superficial $\lambda=620$ (d) images of the same specimen after TB staining the tumor is very dark and can be easily demarcated. The location and shape of the tumor in the images (b), (c), and (d) (arrows) compares well with the frozen H&E section (e) (the red line outlines the tumor margins). A detailed examination shows that in the superficial image acquired before staining and in the conventional image of the stained specimen the tumor appears as a single nest, whereas the superficial image of stained tissue reveals three closely seated tumor lobules. The frozen H&E section (e) confirms that the number and location of the tumor lobules were identified accurately in the image (d) and proves that PLI enables imaging of the superficial tissue layer only. Bar: 1 mm.
PLI examination of nonmelanoma cancers To evaluate the potential of the proposed technique we imaged several types of skin tumor excisions and compared the results to histopathology of the respective tumor. Polarized light images were diagnosed independently of the histologic images. In total, 45 skin excisions obtained from 38 patients were imaged. Thirty-five out of 45 samples were stained with TB (dye concentration in solution varied from 0.1 to 0.5 mg per ml) and 10 samples were stained with MB (dye concentration in solution varied from 0.15 to 0.5 mg per ml). In 41 cases the location and shape of the tumors identified using PLI correlated well with H&E frozen sections. In three cases (two superficial BCC and one SCC in situ; all three specimens were stained using MB with dye concentrations of 0.25 mg per ml, 0.5 mg per ml, and 0.25 mg per ml, respectively) PLI located the tumors correctly, but the size of the lesions was larger in comparison to histopathology. In one case (superficial BCC, stained using TB with dye concentration of 0.1 mg per ml) PLI identified two more tumor nests than histopathology.

In addition to the images presented and discussed above, several more example images of the different types of tumors, stained with TB or MB, acquired at 600 nm or 620 nm, are shown in comparison with histopathology in Figs 8–10. These are the images of infiltrative BCC, stained in TB solution with a dye concentration of 0.2 mg per ml and imaged at 600 nm (Fig 8); nodular BCC, stained in MB solution of 0.2 mg per ml and imaged at 620 nm (Fig 9); and moderately differentiated SCC, stained in TB solution of 0.1 mg per ml and imaged at 620 nm (Fig 10). These representative images demonstrate that dye-enhanced PLI allows accurate and reliable examination of different types of nonmelanoma cancers.

**DISCUSSION**

The results of this *in vitro* study show that dye-enhanced multispectral PLI may be a suitable technique for intraoperative assessment of nonmelanoma skin tumor margins. The images presented in Figs 5 and 6 demonstrate how a multi-wavelength approach enables differentiation of the tissue areas stained with blood from those stained with the dye. An effective discrimination of the chromophores could be achieved because the absorption of the hemoglobin and the dyes (MB and TB) attain their respective maxima in different spectral domains (see Fig 2). A multispectral approach would be especially valuable for examination of specimens with high blood content, as well as for *in vivo* application of this technique.

Staining of the tissue with MB or TB significantly enhanced the contrast of the tumors in the image (see Figs 5–10). Although some healthy structures, such as hair follicles, retain some dye (see conventional images of stained excisions in Figs 6 and 7), the concentration of the dye in the cancerous tissue is much higher.
Consequently, tumor-a¡ected areas appear much darker when imaged with light within the 600–620 nm range. The results of this study suggest that all the investigated wavelengths, which are within the absorption band of TB and MB (i.e., 600 nm (Fig 8a), 610 nm (Fig 6c), and 620 nm (Fig 7d)), provide comparable and high contrast of the tumor in the image. Therefore in a clinical setting it might be possible to reduce the total acquisition time by reducing the number of wavelengths employed for imaging.

As was mentioned above, the combination of tissue staining with polarized light limits the imaged volume to the super¢cial tissue layers and ensures that su⁄cient contrast can be achieved for reliable differentiation between tumor and surrounding tissue. In the wavelength range of considerable dye absorption the section thickness of skin images is approximately 150 μm, which enables a comparison of the super¢cial images with the histopathology (see Figs 5–10). The processed images are remarkably similar to standard MMS maps. Hair follicles, sebaceous glands, fat, and normal stromal elements are visible in detail, and with a di⁄erent appearance from the tumor, which appears very dark due to the increased, in comparison to normal tissue, uptake of the dye.

From 45 skin excisions investigated, in 41 cases PLI correlated well with H&E frozen sections. In three cases (two super¢cial BCC and one SCC in situ) PLI located the tumors correctly but the size of the lesions in the image was larger in comparison to histopathology. In one case (super¢cial BCC) PLI identi¢ed two more tumor nests than histopathology. There are several possible reasons for the discrepancies. First, the section thickness (or imaging depth) in PLI is in the range of 150 μm, whereas histologic H&E sections are 5 μm thick, which could explain why we see larger tumor volume in PLI in comparison to histopathology. Another possible reason could be the damage of cell membranes due to freezing/thawing artifacts that could cause leakage of the dye into the surrounding tissue.

The results of this study indicate that multispectral dye-enhanced polarized light macro imaging can accurately delineate the margins of di⁄erent types of nonmelanoma cancers, including the morphea-form BCC. The lateral resolution of our instrument is approximately 30 μm, and the section thickness of the super¢cial images in the red wavelength range is approximately 150 μm. A specimen 2.8 cm × 2.5 cm in size can be imaged and the image processed in less than 1 min. A clinical trial with fresh specimens is under way to evaluate an optimal dye (TB versus MB), optimize the staining technique, and determine the minimal number of wavelengths required, in order to develop the multispectral PLI technique into a reliable, accurate, and time-e⁄cient method applicable for tumor demarcation in clinical

Figure 9. Nodular BCC (site: cheek). Comparison of (a) the superficial image Iσ620 (λ = 620 nm, section thickness ≥ 150 μm, stain MB) and (b) a histologic frozen section prepared during Mohs surgery (section thickness ≥ 5 μm, stain H&E). Tumor margins in (b) as determined by the Mohs surgeon are outlined with a red line. General morphology of the tumor in the image compares well with that identi¢ed in the histopathology. Bar: 1 mm.

Figure 10. Moderately differentiated SCC (site: ear). Comparison of (a) the superficial image Iσ620 (λ = 620 nm, section thickness ≥ 150 μm, stain TB) and (b) a histologic frozen section prepared during Mohs surgery (section thickness ≥ 5 μm, stain H&E). Tumor margins in (b) as determined by the Mohs surgeon are outlined with a red line. General morphology of the tumor in the image compares well with that identi¢ed in the histopathology. Bar: 1 mm.
practice. The rapid acquisition of these images during surgery can potentially allow tumor removal to progress without taking time to process frozen sections, the most time-consuming step in MMS. A layer of tumor can be removed, the surgical bed can be imaged, residual tumor can be detected, and guided tumor removal can take place.

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REFERENCES


