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BRIEF REPORT

Confocal laser scanning microscopy—evaluation of native tissue sections in micrographic surgery

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Abstract Micrographic surgery is mainly used for excising basal cell carcinomas in high-risk body sites. The time-consuming process of frozen histopathology could be potentially accelerated by the application of ex vivo confocal laser scanning microscopy (CM). We examined the margins of 52 excised basal cell carcinomas by means of CM, and compared the results to conventional histological findings. For contrast enhancement, tissues were treated with 10% citric acid. The morphologic features of tumors seen in CM corresponded well to conventional light microscopy. Confocal assessment and light microscopical findings were consistent in 84.6%. If a tumor is clearly detectable by means of CM, the procedure of micrographic surgery can be accelerated. Negative confocal findings have yet to be confirmed by conventional microscopy. Applicability of CM is limited because of an inconsistent image quality that does not allow a reliable detection of small tumor nests. Further research into the handling of the samples and into specific contrast enhancement is necessary for CM to become a widely accepted procedure in micrographic surgery.

Keywords Basal cell carcinoma · Confocal laser scanning microscopy · Micrographic surgery

The confocal microscope was loaned by Mavig GmbH, Germany

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Introduction

Basal cell carcinomas represent the most common malignant tumor in the USA, Australia, and Europe. In the USA, 1 million new basal cell carcinomas were diagnosed in 2005, and the incidence of this skin tumor continues to grow by 3–10% per year. The most important risk factors are fair skin color and increased exposure to UV radiation. As a result, the highest incidence is found in Australian men, with up to 2,074 cases per 100,000 inhabitants [1]. Sun-exposed body sites are most frequently affected: In up to 85% of patients, lesions are located on the head or neck. Particularly facial basal cell carcinomas have to be removed with a maximum preservation of healthy tissue for reconstruction purposes. These tumors are usually excised by micrographic surgery followed by a complete examination of tissue margins, which results in an optimal low recurrence rate [2].

However, this procedure is rather time-consuming because after each excision, tissue has to be fixed or frozen, embedded, sectioned, and stained. Confocal laser scanning microscopy allows the histological examination of tissue in its native state, as it can generate virtual optical sections throughout the specimen.

In a confocal laser scanning microscope, a laser beam is maximally focused in the focus plane of the specimen and scans an object point by point in two dimensions. Light reflected from this focus is then imaged through a pinhole onto a light detector. The parts of the signal deriving from planes above or below the focus plane are thus suppressed, and the result is a black-and-white real-time tomography with high axial and lateral resolution (‘optical sectioning‘). The focus of excitation and the focus of detection are "confocal" to each other, i.e., they are placed on top of each other. The depth of imaging is 200–350 μm within the tissue, and the scan field of view is 500 × 500 μm.

Refractive index differences of cells, organelles, and microstructures cause a contrast that can be enhanced by treating tissue with diluted acid solutions, which causes chromatin to compact and nuclei to appear bright due to significantly increased back-scatter.

Chung et al. showed that confocal images of healthy skin and basal cell carcinoma correspond well to images obtained by conventional light microscopy. In a preliminary clinical study, they compared confocal images of 92 basal cell carcinomas to conventional Mohs histopathology and found a positive correlation in 69 cases. They could demonstrate that this new technology can be used to evaluate margins of Mohs surgery, whereas it cannot yet substitute frozen histology because some difficulties remain in identifying infiltrative basal cell carcinomas or small nests of tumor cells [3].

We also analyzed the application of confocal microscopy in micrographic surgery by use of an alternative method of micrographic surgery. We examined 52 specimens (primary or subsequent tumor excisions) with CM and compared the results to conventional histological examination with a light optical microscope.

Materials and methods

Tissue collection and preparation

Micrographic surgery was performed as follows [2, 4]: The tumor was excised en bloc, which created a nearly cylindrical sample containing the epidermis, dermis, and subcutis. Then, margin strips were cut off according to the margin strip method (‘Tübingen torte‘) (Fig. 1) [2]. After confocal examination of the outer surfaces of these margin strips, we prepared hematoxylin and eosin-stained frozen sections, which were examined under light microscopy as usual. The undersurface of the sample was analyzed by confocal microscopy, and the remaining sample was then sectioned horizontally, beginning from the undersurface.

Confocal laser scanning microscopy

We used a commercially available confocal laser scanning microscope (VivaScope 2500; Lucid Inc., Rochester, NY). A near-infrared diode laser at 830-nm wavelength served as a light source. The operating optical power was up to 16 mW. A 30× magnifying water immersible objective lens with a numerical aperture of 0.9 was used, allowing an optical vertical resolution of less than 5 μm.

For tissue fixation, the VivaScope was equipped with two identical metal-framed glass windows. One served as an examination slide, whereas the other was used as a cover slip that was fixed to the stage by two metal clips.

The uneven pressure exerted by these metal clips on the cover slip often results in insufficient contact between the tissue and the examination slide, particularly in irregularly shaped tissue samples. Since compression effects and air bubbles can make confocal imaging impossible, we developed a slide on which the cover slip was fixed with four adjustable screws, which allowed a stable adjustment of the pressure of support, even in samples of irregular thickness.

The immersion media used between the slides was 0.9% sodium chloride solution.

The scan field of view was 500×500 μm. Using a stepper motor, the tissue cassette can be moved in XY direction to create maps by the VivaScope software called Viva Blocks. A mosaic of 8×8 images displayed a field of view measuring 4×4 mm. The recording of one 4×4 mm Viva Block took about 35 s. Blocks of lower magnification (up to 12× 12 mm) were not useful, particularly because they were more time-consuming (up to 580 s per map), and smaller tumor nests could not be identified. Once depth and laser power (illumination) were adjusted, we imaged the complete tissue

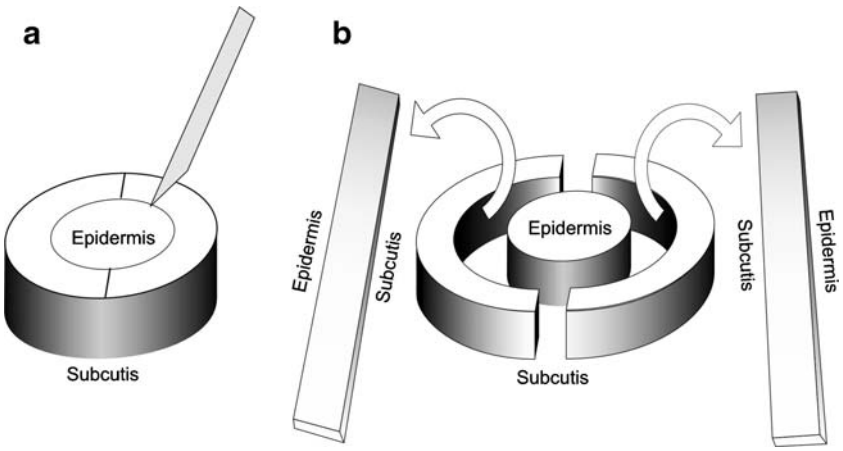

Fig. 1 The margin strip method (“Tübingen torte”): After excision, narrow margin strips are cut off (**a**), the outer surface of the specimen is straightened on an examination slide (**b**). Margin strips and center part are first imaged by confocal microscopy and then horizontally sectioned for light optical microscopy

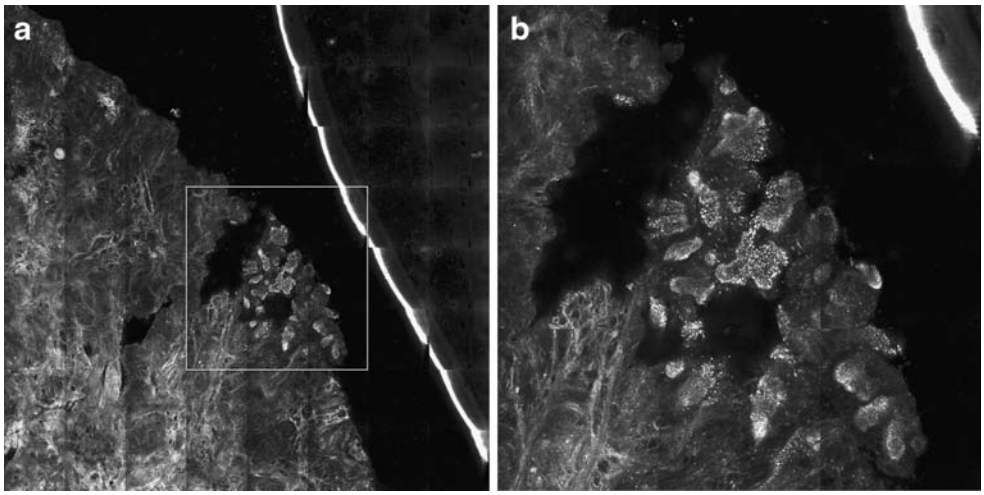

Fig. 2 Map 4×4 mm (**a**), suspect area in higher magnification (1.5×1.5 mm) (**b**)

in Viva Blocks. If suspect areas on these maps were detected, we selected the corresponding image and examined this location with high magnification (Fig. 2).

All images were stored digitally with a resolution of 1,000×1,000 pixels in the BMP file format for the comparison to conventional histology afterwards. The software also stored the examination parameters (laser power...) and comments in a text file format.

As the integrated software of the VivaScope did not permit a comfortable handling of the stored pictures and data, for example a comparison of several images side by side, we used a commercially available media database (MediaDex®; Canto GmbH, Berlin, Germany). A self-programmed Java Plugin connected the database with the VivaScope software.

Tissue preparation

The samples were immersed in 10% citric acid for 60 s and then rinsed with 0.9% sodium chloride. As proven previously, conventional histopathological procedures are not impaired by this treatment [5]. The prepared tissue samples were then placed onto the examination slide as described above.

After the evaluation of the images was made with the confocal microscope, tissue was prepared for conventional light microscopy and analyzed as usual. Subsequently, we compared both histological findings.

Results

We collected 52 specimens from primary as well as from second- or third-stage excisions, i.e., a total of 134 margin strips and undersurfaces were examined.

Morphologic features of basal cell carcinomas seen in confocal images corresponded to those of conventional histology: we found sharply demarcated nests of irregularly arranged and densely packed tumor cells with peripheral palisading. Peritumoral lacunae were also visible. Elongated nuclei appear bright in confocal images, and the nucleus-plasma-ratio is high (Fig. 3).

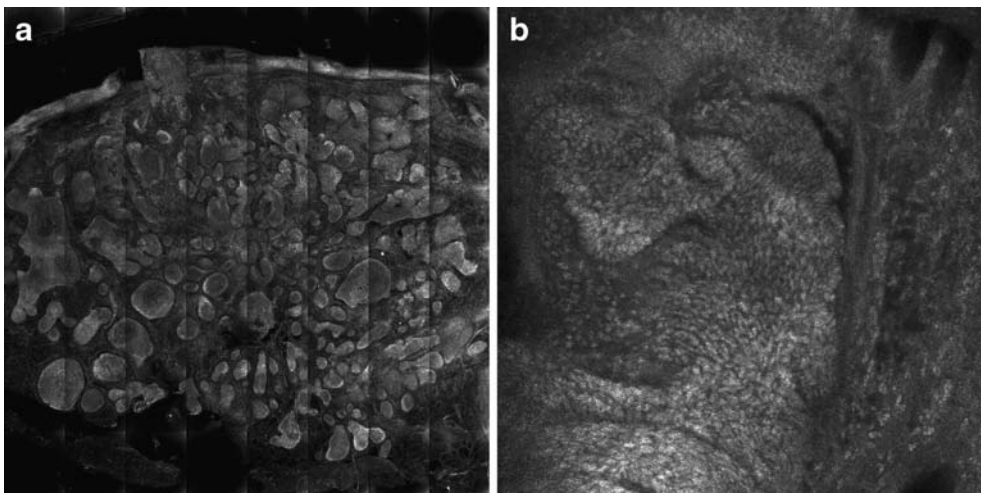

Fig. 3 Nests of tumor cells (map 4×4 mm) (**a**), peripheral palisading and peritumoral lacunae (0.5×0.5 mm) (**b**)

Table 1 Results according to histology

Cases (=excised tumors acc. to Mohs stages)	Correct		Not correct		
	True-positive	True-negative	False-positive	False-negative	Tumors with false-positive and false-negative assessed margins
Nodular	8	8		2	1
Nodular/cystic		2			
Nodular/infiltrative	1	3	2		
Infiltrative	5	12	1	2	
Micronodular tumor		1			
Scar without tumor		1			
Trichofolliculoma		2			
Sweat gland carcinoma		1			
Total	14	30	3	4	1
Sum of cases	44		8		

Sometimes the examination plane needed to be changed dynamically to distinguish cancerous cells from other structures.

The time needed for preparation and imaging was about 6 min, depending on the size of the excised tissue: after treatment with citric acid for 60 s, it took another minute to place the sample on the slide, to fix the slide onto the stage, and to adjust the XY position, laser power, and depth. For example, the recording of the 4×4-mm maps required 3.5 min in a piece of tissue measuring 12×8 mm.

In 44 tumors (or rather excision stages) confocal assessments corresponded to light microscopical findings, which equates to 84.6%. Out of these 44 samples, 30 were true negative and 14 true positive. In one case, one margin was assessed false-negative, whereas another margin of the same tumor was false–positive. Analyzing the 134 margin strips and undersurfaces, we found accordance in 122 samples (23 true-positive, 99 true-negative). The detailed results including the histology of the tumors are given in Table 1.

Four specimens were diagnosed as false-positive. In three cases, hair follicles could not be distinguished from tumor cells. In one sample, a squeezed sebaceous gland was taken for a basal cell carcinoma (Fig. 4). However, correct diagnosis was difficult even in conventional histology.

Five basal cell carcinomas were not detected in confocal images. Sometimes, the cut surface of the sample was not flat. This resulted in incomplete contact with the slide, which disallowed an imaging of the whole surface. In other cases, the image quality was not good enough to distinguish the nuclei of basal cell carcinomas. Three nodular and two infiltrative basal cell carcinomas were among the tumors not detected by confocal microscopy.

Discussion

In this study, we examined the application of confocal laser scanning microscopy in the routine assessment of margins

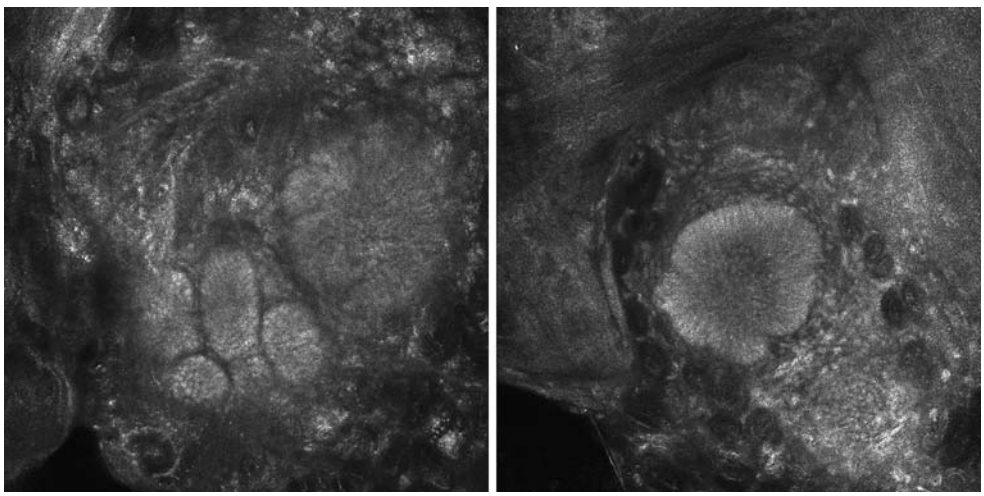

Fig. 4 Two examples of squeezed sebaceous glands that were taken for a basal cell carcinoma (0.5×0.5 mm)

during micrographic surgery by use of a modified margin strip method ('Tübinger Torte'). Although histological characteristics of basal cell carcinomas found in confocal microscopy corresponded well to those seen in conventional light microscopy, this new technology cannot replace frozen histology at present, since a rate of only 84.6% correct results is unacceptable.

The tissue fixation device provided by the Viva Scope allows the imaging of the underside of very flat samples without problems, whereas lateral margins cannot always be pictured: tilting and squeezing impede correct confocal examination. Also, if the cutting surface is irregular, the contact between the tissue and the slide may be incomplete. This problem concerns particularly second- or third-stage excisions. We tried to resolve these problems by means of the aforementioned self-developed slide mount, but particularly in specimens of irregular thickness, imaging the entire surface was not always possible. Consequently, not all areas are imaged at the risk of not detecting nests of basal cell carcinoma.

Another limitation is the inconsistent image quality. Generally, we obtained sharp images, in which cell nuclei could be precisely delineated. In several cases, however, focusing was problematical and the attainable image quality did not allow a differentiation between tumor and benign cells, particularly of follicles and sebaceous glands. The detection of infiltrative basal cell carcinomas requires optimal focusing and image quality, as these tumors often have small infiltrating strands consisting of only a few tumor cells.

Further development of this promising technology should be focused on the handling of the sample, especially the construction of the slide. Patel et al. [6] constructed a sample stage with an adjustable tilt and an adjustable distance of the slide to the objective lens by means of spring-loaded thumbscrews. Constant pressure and flattening of the undersurface of the sample are achieved with a piston and by bolstering the tissue with 3% agarose gel pads.

Certain detection of tumor cells implies a constant high image quality. Particularly the delineation of follicles could be ameliorated by applying a contrast enhancer specific for basal cell carcinomas. However, such a device has yet to be developed. First such developments were reported by Tannous, Torres, and Gonzalez, who found a higher uptake of aluminum chloride by the nuclei of tumor cells compared to normal surrounding skin [7]. Al-Arashi, Salomatina, and Yaroslavsky [8] found that the detection of basal cell carcinomas could be advanced by fluorescence polarization imaging of toluidine or methylene blue-stained samples. This method seems to be particularly interesting with regard to in vivo confocal reflectance microscopy because methylene and toluidine blue are non-toxic substances. However, a possible improvement compared to the staining with acid solutions, which is currently used in ex vivo examinations, has yet to be shown. Recently, Gareau et al. described the use of acridine orange in fluorescence confocal microscopy as a specific contrast agent for basal cell carcinomas [9]. Contrast enhancement could also be obtained by application of cross-polarization, which causes the darkening of collagen in the papillary dermis [6]. Additionally, the examination of large tumors could be accelerated by a field of view that is larger than the presently available device measuring 500× 500 μm.

Conclusions

Facing the present technological state, confocal laser microscopy cannot replace conventional histology for micrographic surgery, since it cannot detect all tumor cells. Nevertheless, in case of clearly detected basal cell carcinoma in the margins, the next surgical stage can be performed within a few minutes. Negative confocal findings have to be confirmed by conventional microscopy, which is not impaired by the preceding non-invasive confocal imaging.

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