Routine application of ex-vivo confocal laser scanning microscopy with digital staining for examination of surgical margins in basal cell carcinomas

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Introduction

Basal cell carcinoma (BCC) is one of the most common tumors in Central Europe, and its incidence is rising [1, 2]. Excision of the tumor is the therapeutic gold standard. This can be performed with various methods [3]. The methods used in this study were either ‘La Galette’ excision with complete margin control, or elliptical excision. The former, in particular, shows good results with low recurrence rates [4]. Histopathological diagnosis is typically performed on the day after surgical intervention using paraffin-embedded slices stained with hematoxylin-eosin (HE). Frozen-section analysis is available within 30 minutes, but its quality is somewhat inferior. It also uses up part of the tissue which subsequently is no longer available for conventional histopathological procedures. Both methods are time and labor intensive, since several trained professionals are needed for preparing the histological slices. Another more recently introduced method is rapid lump examination, which assesses the freshly excised tissue using a stereo microscope [5]. If the margins are tumor-free, the wound can be closed. If tumor cells are detected in the margin, excision is repeated until no tumor cells remain in the margins.

Confocal laser scanning microscopy (CLSM) offers high-resolution tissue scanning within a few minutes. A near-infrared laser beam is applied to the specimen via an intermediate optical system. Light from the laser beam reflected by the tissue is read by a detector. This method is used in...
as a non-invasive ‘optical biopsy’ for diagnosing various skin tumors such as basal cell carcinoma, lentigo maligna, or squamous cell carcinoma [6–11]. One drawback is that in contrast to conventional histology, the section plane of in vivo CLSM is horizontal. Assessment thus requires some experience. Additionally, due to scattering effects, the signal cannot penetrate the skin by more than 250 μm at most. In ex-vivo use, these drawbacks do not apply since the excised specimens can be tilted by 90° and the surface of the surgical margins investigated. Ex-vivo CLSM thus displays the familiar vertical slices just like conventional histological specimens.

Apart from tissue reflection, ex-vivo CLSM can also be combined with fluorescent nuclear staining using acridine orange or proflavine [12, 13]. Newer appliances combine tissue reflection and fluorescent staining in one microscopic image. These images are green (fluorescence), white (reflection), and black (background). Acridine orange staining has no influence on subsequent HE staining [12].

Several studies [9, 11, 13–25] have confirmed the high sensitivity and specificity of this method in diagnosing various diseases, in particular basal cell carcinoma.

HE staining is a standard diagnostic method for basal cell carcinoma since it is easy to use and effective [26].

Digital staining is a new feature that has recently been integrated into ex-vivo CLSM. Automated digital image processing offers real-time on-screen color correction of the CLSM images, showing colors similar to HE staining instead of the less familiar green, white and black images. This is achieved via the similarity of tissue reflection with eosin staining and replacement of the nuclear hematoxylin staining by acridine orange fluorescence [27]. Digital HE staining has already been comprehensively evaluated in healthy skin samples [28].

The aim of our study was a prospective evaluation of ex-vivo CLSM with digital HE staining for assessment and surgical margin examination in basal cell carcinoma. We investigated the basic suitability of this method in comparison with conventional histological processing, as well as its practical value in day-to-day clinical practice – especially considering time requirements.

Patients and methods

This study was approved by the University of Leipzig Medical Faculty ethics committee (O93/18-ek). Between July and October 2018, 79 patients with suspected basal cell carcinoma were prospectively enrolled in the study. All participants gave written informed consent. Inclusion criteria were an age of at least 18 years, clinical diagnosis of basal cell carcinoma, surgical excision of the BCC, and provision of written informed consent.

Confocal laser scanning microscopy (CLSM)

The VivaScope® 2500M-G4 (MAVIG Germany GmbH) was used in this study (Figure 1). This microscope combines two lasers with wavelengths of 785 nm (infrared) and 488 nm (blue). The maximum scan area is 25 mm x 25 mm. A camera takes a macroscopic image of the specimen, which allows easy navigation and selection of certain regions within the sample. VivaScope® offers up to 550-fold continuous magnification.

Procedure

For this study, the confocal laser scanning microscope was positioned within the surgery wing. Basal cell carcinomas were excised according to routine protocols: 41 via elliptical excision, and 60 via a modified La Galette procedure with complete margin control (Figure 1a–c) [4]. In this procedure, the tumor itself as well as the margins on the sides and below are prepared separately in situ and then excised, so all margins are cut in preparation for histological processing, and complete margin control is ensured.

The specimens were immersed in 0.9 % NaCl solution immediately after excision to prevent desiccation. Ex vivo CLSM was then performed. Slices of 2–3 mm thickness were cut from the tumor specimen. The lateral margins and the tumor base margin could be evaluated directly on the excision plane.

The specimens were briefly dipped in phosphate buffer (PBS, pH 7.4) and then stained with 0.12 mM acridine orange for 20 seconds. To remove excess color, they were again immersed in PBS pH 7.4 for 10 seconds and then attached to a microscope slide. The specimen was pressed flat on the slide by using a coverslip fixated by re-usable rubber glue (BluTack®, Bostik Inc.; Paris, France).

The specimens were then positioned on the confocal laser scanning microscope. Depth as well as representation of reflection and fluorescence were determined manually, and the scan initiated. The scanning area was specified in a macroscopic image which was also used for navigation. Digital staining was selected from the software menu, and remained unchanged thereafter. In cases where the specimen was larger than the 25 mm x 25 mm scan area, several partial scans were performed – this however only applied to a small number of large skin tumors. The procedure was timeline throughout. Afterwards, the specimens were sent to our histological laboratory for conventional histological assessment of paraffin-embedded slices. The results of this conventional diagnostic procedure were available on the next day.

After all images had been obtained, they were evaluated in a blinded manner by an experienced dermatohistopathologist.
(UP) on a large monitor. Both the tumor and the R status were assessed. As with conventional HE staining, retraction artifacts, palisade patterns of the nuclei, nucleocytoplasmatic ratio, and stroma reactions were noted. In cases of discordant findings, a second investigator (MI) was consulted.

**Results**

**Patient cohort**

A total of 109 excised tumors from 79 patients (29 female and 50 male) were entered in this study. The average age of the patients was 73.2 years (Table 1). 101 basal cell carcinomas were identified and included for assessment. 60 of these had been excised via the modified La Galette procedure with total margin control [4], and 41 via elliptical excision. Altogether, 409 specimens were evaluated.

Eight excised tumors were excluded from the study. Five of these proved to be tumors other than basal cell carcinoma (Bowen’s Disease, and squamous cell carcinoma). Two specimens could not be evaluated due to excess ink from the pre-excision marking, and one result was accidentally deleted by the software and could not be retrieved.

**Margin control in basal cell carcinoma**

In the 60 basal cell carcinomas excised with complete margin control, the tumors themselves as well as the surgical margins (ordered according to the clock method) and the tumor bases were assessed. Altogether, between six and eight slices for each basal cell carcinoma excised with this method were scanned with CLSM, amounting to a total of 368 specimens. Preparation of all confocal images for a tumor took 42:13 ± 15:47 minutes on average. The duration of assessment depended on the number of slices, the size of the specimen, and the number of scans required for obtaining the best possible image. The time for assessment per specimen was 6:34 min on average. For the 41 basal cell carcinomas removed via elliptical excision, one transverse section was evaluated per tumor. On average, microscopic evaluation took 14:20 ± 7:30 min from excision. Specimens evaluated later in the study generally required less time than those early in the study, indicating a training effect in handling the specimens and using the software.

A total of 409 results for the tumors and the R status of the surgical margins were obtained. Four results were classified as “not assessable”. The remaining 405 results were...
compared with the results from classic histology (Table 2, Figures 2, 3).

**Evaluation of the conflicting results**

Out of a total of 405 specimens, 34 findings showed conflicting results in CLSM as compared with histology, 11 false-positive and 23 false-negative. Re-evaluation of the 11 false-positive results in a direct comparison of the images showed that sebaceous glands (n = 4), inflammatory lymphocytic infiltration (n = 3), epidermal cones (n = 1), or hair follicles (n = 1) had erroneously been diagnosed as tumor cells. Two specimens were evaluated incorrectly without an identifiable cause (Figure 4).

The cause of the 23 false-negative findings was insufficient quality of the scans. In most cases, the epidermis was not completely visible, or parts of the specimen had accidentally been folded over, so that for example superficial tumor cell nests were overlooked.

In 13 other cases of conflicting results, the excision had been performed with very narrow margins in healthy tissue. The true surgical margins (the cuts evaluated in ex vivo confocal imaging) were tumor-free, but after the surgical margins had been prepared for paraffin-embedded histology with step-wise grading in the direction of the tumor, tumor cells were detected. According to the routine procedures for frozen section analysis, the areas were comprehensively re-evaluated and the images compared side by side. They were subsequently considered ‘true negative’ (with very narrow excision in healthy tissue).

**Qualitative evaluation**

Large specimens had to be assessed in several sections. Since the fluorescence of acridine orange fades over time, the second part of the scan usually appeared paler (Figure 5). Manually adjusting the software setting can usually compensate for this effect, at least partially.

We also noticed that the ink used for marking the outer margins had a negative effect on acridine orange staining. Thus, at the beginning of the study, the practice of marking the outer margins had to be changed and only a tiny dot of ink was applied to the sections.

**Discussion**

Our study shows that confocal laser scanning microscopy (CLSM) with digital HE staining is very well suited for detecting basal cell carcinomas and evaluating the surgical margins. Basal cell carcinomas are easily distinguishable from other skin structures. So-called retraction artifacts and palisade patterns of the cellular nuclei in the marginal areas can be clearly detected. This has already been shown in earlier studies for appliances working without digital HE staining [13–15, 19]. Digital staining imitates the characteristic basophil staining of cells seen in the familiar HE images. Basal cell carcinomas are easily detectable even without digital staining, but for experienced histologists, this technique...
offers the additional advantage of assessment without changing their familiar viewing habits. All experts involved in our study stated that digital staining offered a clear subjective improvement as compared with the original green/white/black images.

In some cases, limited image quality may lead to problems in differentiating tumor cell accumulations and stroma reactions from lymphocytic infiltration or sebaceous glands. These problems have already been described in several earlier studies without digital staining [17, 19, 23].

Some earlier studies reported higher rates of sensitivity and specificity. This may be explained by several factors: Most studies were not performed under the conditions of routine clinical practice, so positioning of the specimens could be corrected and imaging optimized [16, 18, 23]. In our study, this single factor amounted to 62% of all incorrect results. Under “real world” conditions, corrections when positioning the specimens were less easy. In many cases, the epidermis was not fully visible due to rolling or because it did not completely adhere to the slide in cases of oblique cutting. Air bubbles are another possible cause of inadequate imaging [13, 17, 20]. After data collection for this study had been concluded, the manufacturer introduced a special slide for ex vivo CLSM. Via a foam pad with magnets, the contact

**Figure 2** Basal cell carcinoma. Macroscopic image of a specimen (a). Fluorescence and reflection (b). Digital H&E staining (c). Paraffin-embedded slide section (d).

**Figure 3** Magnification of a sample: overview (a), medium magnification (b), high magnification (c).
pressure can be distributed evenly over the whole specimen. We expect that this will lead to improved detection of all surgical margins and thus decrease the rate of false-negative results.

One other aspect is the rate of true positive results: This rate was comparatively low (87/405) in our study, due to the cutting of the specimens with total margin control (Table 2). The positive and negative predictive values thus offer only limited comparability.

Only two previous studies have compared ex vivo confocal laser scanning microscopy with histopathological paraffin slices. The working group from Tübingen [13] used comparable techniques for excision and margin control of basal cell carcinomas, but another laser scanning microscope (Histolog® Scanner, Samantree). They studied 525 surgical margins in a clinical setting and found almost identical values for sensitivity (73 %) and specificity (96 %) as we found in our study. Another working group with extensive experience

**Figure 4** Confocal images of suboptimal quality: Sebaceous glands (a) and inflammatory infiltrates (c) were sometimes difficult to distinguish from BCC tumor nests. Corresponding conventional histopathological images (b, d).

**Figure 5** Large specimen that required scanning in two parts. First scan (a), second scan with notably faded fluorescence signal (b).
Ex-vivo CLSM with digital staining in basal cell carcinoma

in confocal laser scanning microscopy [14] analysed a total of 753 surgical margins of basal cell carcinomas in a study setting. They found a somewhat higher sensitivity (89.7 %) compared to our results, while their value for specificity was 95.3 % and thus similar to ours. This suggests that apart from initial learning curves, time pressure does have an influence on the quality of confocal laser scanning microscopy.

Learning curves for confocal laser scanning microscopy have been described elsewhere [13, 16, 23, 24]. Even though investigators needed only a two-day introduction to use the appliance with confidence, we did notice that image quality improved over the course of our study, and there was a trend towards requiring shorter periods of time.

The scan area was 25 mm x 25 mm and thus relatively large as compared with older appliances. Yet it was insufficient for tumors larger than these dimensions. These specimens need to be scanned in two or even more sections, which not only requires much more time but may also deteriorate image quality due to decreasing fluorescence of the acridine orange. This is a particular drawback in case of large elliptical excisions. Excisions according to the abovementioned modified La Galette procedure with complete margin control did not pose this problem since the size of the surgical margins can be chosen.

The appliance has been designed for use in day-to-day clinical practice. It is relatively small and can be placed within the surgical wing without occupying too much space. It is, however, important to avoid vibrations since this would require re-calibration.

The ex-vivo CLSM images are digital, and assessment by a (dermato)pathologist can be performed anywhere. This is a great advantage.

It should, however, be noted that the images comprise several gigabytes and thus generate large amounts of data. Our study generated a total of about 3.5 TB, which quickly exceeded the computer’s memory capacity and required external data storage. German law demands that images from routine diagnostics be stored for ten years, just as histological slices in pathology, so appropriate storage capacity needs to be provided.

Improved procedures, such as easier positioning of the specimens, will presumably generate better images in future while retaining speed. As with intrasurgical frozen section analysis, ex-vivo CLSM enables histologically proven R0 resection of basal cell carcinomas with the option of wound closure in a single-step procedure. Infections due to open wounds can be avoided and hospital stays of in-patients reduced. As opposed to frozen section analysis, this can be achieved without loss of tissue. Ex-vivo CLSM with digital HE staining thus has the potential to replace frozen section analysis in the future. On an international level, basal cell carcinoma excision with complete margin control is usually done by Mohs Surgeons who perform both the excision and the assessment of the frozen sections. In this setting, ex-vivo CLSM may one day replace conventional histology assessment.

Ex-vivo confocal laser scanning microscopy with fluorescence-marked antibodies may in future also be used for immune histochemical analyses [29].

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Conflict of interest

The confocal microscope VivaScope® 2500M-G4 was loaned to our Department of Dermatology, Venereology and Allergology free of charge by MAVIG GmbH for the duration of this study.

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