

# Confocal Laser Scanning Microscopy vs 3-Dimensional Histologic Imaging in Basal Cell Carcinoma

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**Objective:** To compare ex vivo confocal laser scanning microscopy (CLSM), which offers rapid images without the need for tissue processing, vs 3-dimensional histologic imaging, the criterion standard treatment for basal cell carcinomas in high-risk areas of the face.

**Design:** Single-center prospective trial.

**Setting:** Dermatological unit of a university hospital.

**Patients:** Seventy-two consecutive surgically removed basal cell carcinomas were examined using CLSM vs standard paraffin-embedded 3-dimensional histologic imaging.

**Interventions:** A total of 312 images, including 73 midsections, 196 lateral margins, 23 “muffins,” and 20 “bread loaf sections,” were obtained using CLSM. Immediately after surgery, the CLSM images were evaluated by the surgeon. The following day, the

3-dimensional histologic slides were evaluated and compared with the CLSM images.

**Main Outcome Measures:** Diagnostic accuracy of ex vivo CLSM to detect tumor strands of basal cell carcinomas and the practicality of using CLSM vs 3-dimensional histologic slides in micrographic surgery.

**Results:** The sensitivity of CLSM reached 94.0% in midsections, 73.7% in lateral margins, 80.0% in muffins, and 80.0% in bread loaf sections. The CLSM images were evaluated by the surgeon within 7.5 minutes.

**Conclusions:** Confocal laser scanning microscopy lacks high sensitivity to detect small tumor strands of basal cell carcinomas. In the future, CLSM may represent a time-saving and less expensive alternative to cryostat histopathologic examination.

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**B**ASAL CELL CARCINOMA (BCC) is the most common type of skin tumor in the world.<sup>1</sup> Principally affected are sun-exposed body surfaces, predominantly the head and neck area.

Excision by micrographic surgery (using 3-dimensional histologic imaging) is the criterion standard treatment for BCCs in high-risk areas of the face, offering precise excision with minimal loss of surrounding healthy tissue. Surgical removal of the tumor is followed by complete visualization of the 3-dimensional excision margins via the cryostat or paraffin technique.

*For editorial comment  
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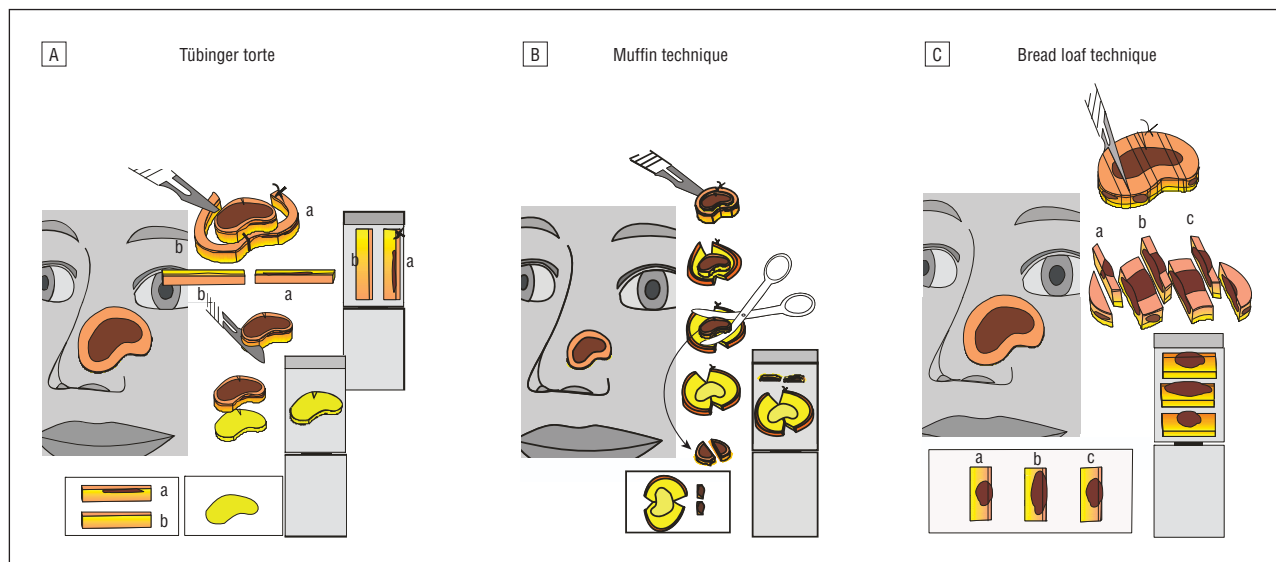
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Germany.

In Mohs micrographic surgery, the excised tumor specimens are sliced, pressed flat on the microtome of a cryostat, and evaluated using frozen sections. If tumor is identified at the deep margin periphery, the pro-

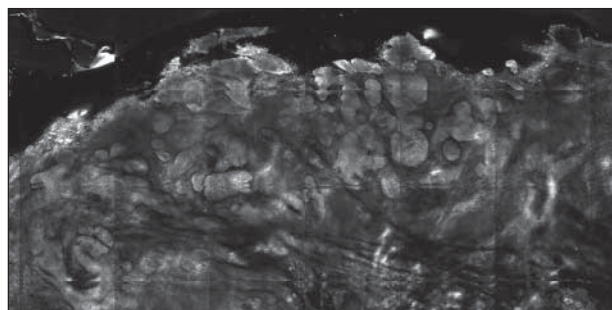
cedure is repeated with another stage of marking and excision. Each stage of removal and examination takes 20 to 45 minutes.

In recent years in Europe, 3-dimensional histologic imaging has gained acceptance as a technique of choice using the Tübinger “torte”<sup>2-5</sup> or the “muffin” method.<sup>6,7</sup> For the Tübinger torte, a narrow lateral strip is excised vertically around the full perimeter of the excised tissue, providing complete visualization of the excised tumor margins. For the muffin, the excised tumor base is placed in the same layer as the margins, providing a complete 3-dimensional view of the lateral excision margins and the base in a single slide. All slides (Tübinger torte and muffin) are embedded in paraffin and then stained with hematoxylin-eosin. Paraffin sections are available within 20 hours. The procedure is repeated until tumor-free margins are obtained.

Ex vivo confocal laser scanning microscopy (CLSM) is a newly developed procedure that may represent an attractive alternative to frozen histologic or paraffin sections.<sup>8,9</sup> Immediately after excision of the



**Figure 1.** Techniques of tissue preparation. A, Narrow lateral strip excised vertically 360° around the full perimeter of the tumor border (a, the 0- to 6-o'clock position; b, the 6- to 12-o'clock position) and a horizontal section taken from the bottom. B, Margins can be folded laterally to the horizontal plane. C, 1-mm sections of fresh tissue, here extracted in 3 steps (a, b, and c). Adapted from the study by Moehrle et al<sup>10</sup> with permission from Wiley-Blackwell, Oxford, England.



**Figure 2.** Confocal laser scanning microscopy mosaic image of a nodular basal cell carcinoma after incubation of the specimen in 10% acetic acid for 90 seconds. Acetic acid incubation before confocal laser scanning microscopy imaging induces compaction of chromatin, which increases light backscatter and renders nuclei detectable.<sup>11</sup>

tumor, CLSM images can be evaluated without the need for tissue processing. The objective of this study was to analyze the potential use of ex vivo CLSM for rapid visualization of excised BCC surgical margins in high-risk areas of the face.

## METHODS

### EXCISION AND STAINING OF TISSUE

Seventy-two consecutive surgically removed BCCs were examined in the Department of Dermatology, University Hospital, Eberhart Karls University in Tübingen, Germany. For 3-dimensional histologic slides, the paraffin technique was used. The tumor specimens were further dissected using the following techniques: Tübinger torte, muffin, and “bread loaf” (using 1-mm step sections)<sup>10</sup> (**Figure 1**).

The Tübinger torte technique involves excision of the tumor and a clinical “safety” margin using vertical or even overhanging incision lines. From the specimen (ex vivo), a narrow lateral strip is excised vertically 360° around the full perimeter of the tumor border, beginning with a topographic mark

at the 12-o'clock position relative to the body axis. The strip is then laid flat and cut into pieces, which are placed in routine cassettes for horizontal fixation. Subsequently, a horizontal section is taken from the bottom of the excised tumor and is likewise prepared flat in a histologic cassette. Routine overnight paraffin fixation is used. This method provides a complete 3-dimensional view of the excision margins.

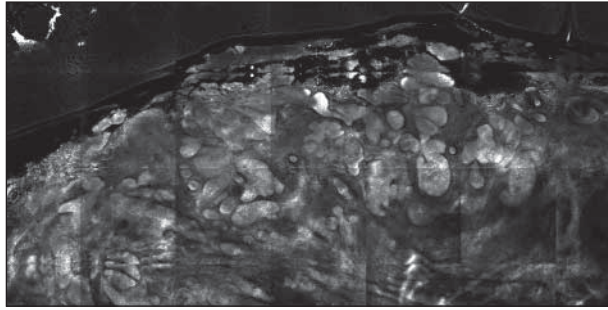
The muffin technique involves excision of the tumor and a clinical safety margin using vertical or even overhanging incision lines. After deep incisions at the 12-o'clock and 6-o'clock positions relative to the body axis, a narrow lateral strip is cut without dissecting the base. Similar to peeling off a muffin's paper mold, these margins can be folded laterally to a horizontal plane. The specimen is placed in a routine cassette for horizontal fixation. The specimens, which are available 20 hours later, provide a complete 3-dimensional view of the excision margins and the base on a single slide. This technique may be used for specimens up to 2 cm in diameter.

The bread loaf technique involves excision of the tumor and a clinical safety margin using vertical or even overhanging incision lines. After topographic orientation of the tumor, 1-mm sections of fresh tissue were extracted and placed in a routine cassette for fixation. The slides were available 20 hours later. All 72 tumor specimens were resected with a safety margin of 2 to 4 mm around the clinically visible tumor.

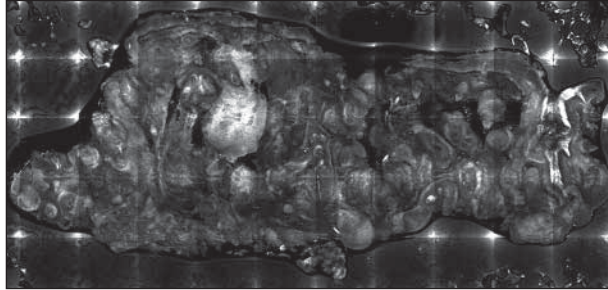
As previously described,<sup>11</sup> incubation of the specimens in acetic acid before CLSM imaging induces compaction of chromatin, which increases light backscatter and renders nuclei bright and easily detectable. After testing different methods (eg, polyvinylpyrrolidone-iodine preparations, eosin, methylene blue, and toluidine blue) to improve staining of BCC specimens, toluidine blue yielded the most precise and clear images (**Figure 2** and **Figure 3**). For this study, tissue was soaked in 10% acetic acid for 90 seconds, stained in toluidine blue for 2 minutes, and imaged by CLSM. After imaging, the specimens were placed in a routine cassette, and tissue was prepared using the standard paraffin method with hematoxylin-eosin staining.

### CONFOCAL LASER SCANNING MICROSCOPY

A modified version of a commercially available confocal laser scanning microscope (VivaScope 2500; Lucid Inc, Rochester, New



**Figure 3.** Confocal laser scanning microscopy mosaic image of a nodular basal cell carcinoma shown in Figure 2 after incubation of the specimen in 10% acetic acid for 90 seconds and in toluidine blue for 2 minutes, providing better contrast.



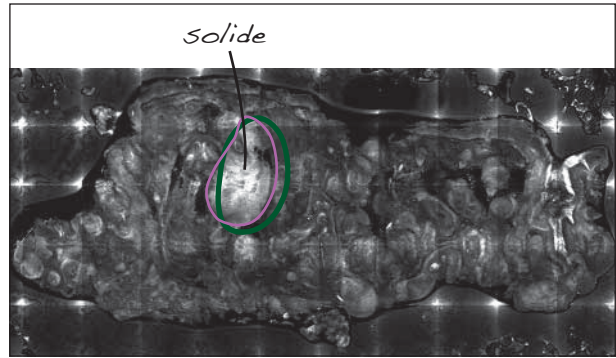
**Figure 4.** Confocal laser scanning microscopy mosaic image of a nodular basal cell carcinoma margin.

York) was used; this version was designed for imaging *ex vivo* excised fresh tissue. The design and instrumentation details for this microscope were reported previously.<sup>8,9,12</sup> The illumination wavelength is 830 nm from a diode laser, with an illumination power of less than 16 mW at the tissue level. An  $\times 30$  magnifying water immersion objective lens (Lucid Stable View, Lucid Inc) with a 0.9 numerical aperture was used, providing a field of view of  $0.5 \times 0.5$  mm<sup>2</sup>. The principle was to obtain a totally flat surface on the cover glass of the inverse microscope without damaging the fresh tissue. For handling large fresh specimens, a  $10 \times 10$ -cm plastic square with a  $4 \times 4$ -cm cavity in the middle filled with cellular material as a hydrophilic foam dressing (Curaform Plus; Kendall Company, Mansfield, Massachusetts) was constructed so the tissue could be pressed without damaging it. Another square of plastic was placed above the cellular material. The square was weighted with 1 to 6 cubes of lead (10-60 g total). Adequate pressure was applied to the cellular material to bring the excision margins into a single flat plane.

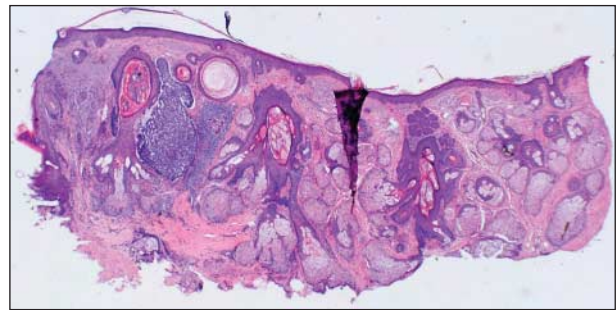
Illumination was automatically adjusted to obtain confocal images. The depth had to be manually adjusted. Because of the confocal field of view (0.25 mm<sup>2</sup>), sequences of images were stitched together to form a confocal mosaic. The imaging of a  $13 \times 11$ -mm mosaic took 150 seconds, and the subsequent stitching required about 30 seconds. All mosaics could be observed directly on the microscope display using the zoom function.

#### COMPARISON OF CONFOCAL MOSAICS WITH CONVENTIONAL HISTOPATHOLOGIC SLIDES

Immediately after surgery, all imaged confocal mosaics of an excised tumor specimen were evaluated by the attending surgeon (H.B., W.S., or M.M.) on the microscope display. All 3 surgeons are experienced dermatopathologists with long-standing experience in evaluating slides of skin tumors and tumor margins in micrographic surgery. They were familiar with CLSM from their work on a 2009 study.<sup>7</sup> Before performing that study, all 3 surgeons had received a formal introduction to in-



**Figure 5.** Corresponding printed mosaic image of slide shown in Figure 6. The tumor detected by confocal laser scanning microscopy was highlighted in red on the printout by the surgeon immediately after operation. The next day, the surgeon received corresponding hematoxylin-eosin-stained paraffin sections and highlighted the tumor island in green on the printout.



**Figure 6.** Hematoxylin-eosin-stained histologic slide of the margin with nodular basal cell carcinoma outgrowths corresponding to the confocal laser scanning microscopy images shown in Figure 4 and Figure 5.

terpretation and evaluation of CLSM images. The imaged mosaics (**Figure 4**) were printed so the BCC and suspicious areas could be highlighted in red on the printout by the surgeon immediately after the operation (**Figure 5**). The next day, the surgeon read the corresponding hematoxylin-eosin-stained histologic slides (**Figure 6**); tumor islands were highlighted in green (Figure 5). The study was approved by the ethics committee of the University Hospital, Tübingen, Germany.

## RESULTS

A total of 312 images, including 73 midsections, 196 lateral margins, 23 muffins, and 20 bread loaf sections, were obtained using CLSM from 72 surgically removed BCCs. These results are summarized in **Table 1**.

#### CLSM FOR RAPID EXAMINATION OF SURGICAL EXCISIONS

On average, the preparation and staining of tissue for CLSM took 4.5 minutes, including 0.5 minute for dissection of the surgical specimen, 1.5 minutes for immersion in acetic acid, 2.0 minutes for toluidine staining, and 0.5 minute for fixation of tissue on the cover glass. Imaging and stitching of  $143$ -mm<sup>2</sup> images required 3.0 minutes at most. Each mosaic was ready for evaluation by the surgeon within 7.5 minutes. In contrast to a previous study,<sup>7</sup> larger images of  $20 \times 20$  mm could be viewed, and the zoom function ranged from  $0.5 \times 0.5$  to  $1.5 \times 1.5$  mm.

## SENSITIVITY AND SPECIFICITY OF BCC DETECTION

The sensitivity and specificity of BCC detection varied across midsections, lateral margins, muffins, and bread loaf sections. The overall sensitivity of CLSM was 94.0% in midsections (**Table 2**). Midsections have a high probability of showing large portions of BCC nests. The overall sensitivity of CLSM was 73.7% in lateral margins with smaller tumor strands. The accuracy of CLSM diagnoses varied based on the section type and the observer. These results are summarized in **Table 3**.

### COMMENT

Three-dimensional histologic imaging with frozen or paraffin histologic examination is time-consuming and expensive. It requires specialized equipment and trained personnel. In contrast, CLSM may represent a time-saving and less expensive alternative for micrographic surgery. With CLSM, images of fresh excised tissue can be obtained and evaluated within 7.5 minutes.

**Table 1. Confocal Laser Scanning Microscopy Results Among 312 Images From 72 Surgically Removed Basal Cell Carcinomas**

Subtype	Midsections (n=73)	Lateral Margins (n=196)	Muffins (n=23)	Bread Loaf Sections (n=20)
Nodular	37	69	3	9
Infiltrative	5	9	0	2
Mixed	9	18	1	0
Superficial	0	3	1	0
Tumor free	19	95	18	9
Histologic slide not evaluable	3	2	0	0

In small<sup>13</sup> and large<sup>7</sup> recent studies, tumors and their margins were evaluated to assess the reliability of CLSM compared with standard histologic examination. Suggested recommendations and technical improvements were incorporated into the present study. The use of midsections, lateral margins, muffins, and bread loaf sections covers a broad spectrum of techniques and reflects the introduction of CLSM into daily surgical routine.

Immersion in acetic or citric acid brightens the morphologic structures of the nucleus in CLSM.<sup>11,13</sup> However, it is insufficient for identification of all BCCs, because small nests of infiltrative BCCs were often overlooked.<sup>7,8,13</sup> Toluidine blue is not considered 100% cancer specific but has been used in clinical practice for staining various carcinomas in vivo.<sup>14</sup> With this staining method, considerably higher contrast of tumor cells compared with normal dermis is obtained, which likely explains the improved sensitivity herein compared with previous results.<sup>7</sup> The nucleus and tumor structure are more easily identified. However, except for midsections, sensitivity herein for margins did not reach 90%, which would be required to replace the current method of standard histopathologic examination.

Because of technical improvements, a complete mosaic of 312 confocal images can be observed directly on the microscope display. For the first time, to our knowledge, we were able to integrate CLSM as a standard procedure in daily surgical routine. Images were available immediately after surgery. Therefore, we were able to evaluate specimens at the bedside of the patient to decide immediately about further treatment.

Another new development in CLSM is a zoom function, which allows closer view of the specimen up to a field of view ranging from 0.5 × 0.5 to 1.5 × 1.5 mm<sup>2</sup>. In the horizontal imaging direction, the stitches overlapped by 1% to 2%, which aided in correct interpretation of the images. However, in the vertical imaging direction, the stitches overlapped by 8% to 10%, which made

**Table 2. Accuracy of Confocal Laser Scanning Microscopy in Detecting Basal Cell Carcinomas**

Variable	%					
	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	False Positive	False Negative
Midsections (n=69) <sup>a</sup>	94.0	36.8	79.7	70.0	63.2	6.0
Lateral margins (n=190) <sup>a</sup>	73.7	69.2	70.7	72.2	30.9	26.3
Muffins (n=23)	80.0	61.1	36.4	91.7	38.9	20.0
Bread loaf sections (n=19) <sup>a</sup>	80.0	77.8	80.0	77.8	22.2	20.0

<sup>a</sup>The area of the confocal laser scanning microscopy image did not correspond to the hematoxylin-eosin-stained slide because of technical artifacts in 4 midsections, 6 lateral margins, and 1 bread loaf.

**Table 3. Accuracy of Confocal Laser Scanning Microscopy Diagnoses Based on Rates of the Individual Observers**

Observer	%					
	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	False Positive	False Negative
1 (n=127)	71.4	66.1	69.2	68.4	33.9	28.6
2 (n=62)	86.1	45.8	70.5	68.8	54.2	13.9
3 (n=123)	86.9	70.2	75.7	83.3	29.8	13.1

a correct evaluation more difficult. Therefore, stitching quality needs improvement.

A complete 13 × 11-mm mosaic could be obtained in less than 3 minutes. Compared with previous studies<sup>15,16</sup> of CLSM in which at least 9 minutes was required, this is a tremendous increase in imaging speed. Another time-saving factor was the automatic brightness and contrast adjustment before imaging.

It was challenging to quickly obtain a flat surface of fresh excised tissue without damaging it. In a 2009 study,<sup>17</sup> tissue was frozen and cut for cryostat-sectioned margin control, with subsequent creation of CLSM images. That procedure was easier but did not evaluate the true outer surface of the excisions. The "histosurgical" flattening of the specimen increased the risk of false-positive results. With the tissue-mounting method used in this study, a flat surface was obtainable for almost all sizes and thicknesses of fresh excised tissue and avoided the freezing step, which is undesirable at the bedside.

As aforementioned, all surgeons herein had similar knowledge and experience in evaluation of normal histopathologic slides and CLSM images. However, the sensitivity and specificity results of these slides and images varied considerably. Because of the immediacy of evaluations during or between surgical procedures, factors such as time pressure, stress, or fatigue could have influenced the results. Variation in results among the 3 surgeons may reflect the real-life setting of the study. Unfortunately, the 3 surgeons had no further experience with CLSM between the previous study<sup>7</sup> and the present study, which made interpretation of the first cases herein more difficult. A "refresher" introduction might have been helpful. Future investigations may focus on the ability of pathologists to evaluate confocal images. Digital CLSM images could be sent from the surgical theater to a pathologist trained in interpretation of CLSM images, enabling surgeons without specific dermatopathologic training to perform rapid micrographic surgical procedures.

There was continuous improvement in handling and fixing the fresh tissue. This factor might have influenced the interobserver variability. However, BCC identification on images of micrographic surgery specimens did not reach the desired rate. In recent studies,<sup>15,17,18</sup> a fluorescent marker was used in CLSM, which facilitated BCC identification on confocal images and resulted in high specificity. These studies were performed in a laboratory and not in a clinical setting. Specific fluorescence could be used in combination with the microscope used herein, offering fast imaging and correct interpretation directly in the operating theater. This would represent a revolution in micrographic surgery.

In conclusion, CLSM lacks high sensitivity to detect small tumor strands of BCCs. Training surgeons to read CLSM images is essential. For micrographic surgery, CLSM may represent a time-saving and less expensive alternative to cryostat histopathologic examination.

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**Author Contributions:** Ms Ziefle and Dr Moehrle had full access to all the data in the study and take responsibility

for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Breuninger and Moehrle. *Acquisition of data:* Ziefle, Schüle, Breuninger, Schippert, and Moehrle. *Analysis and interpretation of data:* Ziefle, Schüle, and Moehrle. *Drafting of the manuscript:* Ziefle, Schüle, and Moehrle. *Critical revision of the manuscript for important intellectual content:* Breuninger and Schippert. *Obtained funding:* Moehrle.

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