Basal cell carcinoma (BCC) is a form of skin cancer originating from the basal cell layer of the epidermis and associated follicular structures. It is the most common human cancer, accounting for 25% of all cancer cases and 75% of skin malignant neoplasms diagnosed in the United States. More than 2 million new nonmelanoma skin cancers are estimated to have been diagnosed in United States in 2014. Basal cell carcinomas are diagnosed primarily by clinical evaluation. Furthermore, an increasing number of clinicians are being trained in the art of dermoscopy to aid in the diagnosis of early tumors. The definitive diagnosis has always been provided by a skin biopsy followed by sample preparation and histopathologic examination.

Advances in optical imaging and spectroscopy technologies raise the possibility of performing rapid and noninvasive light-based histopathologic examination, a pain-free process that would be appreciated by patients and reduce the time from consultation to treatment. Therefore, technologies such as conventional optical coherence tomography (OCT), high-definition OCT (HD-OCT), reflectance confocal microscopy (RCM), multiphoton microscopy (MPM), fluorescence lifetime microscopy (FLIM), and multimodal spectral approaches have been investigated for their applicability for diagnosis of skin cancer, including BCC. Each of these techniques has strengths and limitations that at present make them complementary.

Both OCT and HD-OCT have the benefit of deeper penetration (several hundreds of microns) compared with the microscopy techniques. However, although HD-OCT offers improved resolution compared with conventional OCT, it still proves insufficient in the assessment of the cellular morphologic characteristics in tumor nests, which is important for accurate diagnosis of BCC. The microscopy laser-scanning tech-
Table. Location of the Lesions, Pathologic Diagnosis, and MPM Morphologic Features of BCC

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Location</th>
<th>Pathological Diagnosis</th>
<th>Morphologic Features</th>
<th>Elongated Tumor Cells Aligned in 1 Direction</th>
<th>Parallel Collagen and Elastin Bundles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thigh</td>
<td>SBCC</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Shoulder</td>
<td>SBCC + NBCC</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Arm</td>
<td>SBCC</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Shoulder</td>
<td>SBCC</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>Back</td>
<td>SBCC</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Back</td>
<td>NBCC</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>Face</td>
<td>MNBCC + NBCC</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Leg</td>
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<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
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</tr>
<tr>
<td>10</td>
<td>Arm</td>
<td>SBCC</td>
<td>+</td>
<td>–</td>
<td>–</td>
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</table>

Abbreviations: BCC, basal cell carcinomas; MNBCC, micronodular BCC; MPM, multiphoton microscopy; NBCC, nodular BCC; SBCC, superficial BCC; +, present; −, absent.
In MPM, TPEF derives its signal from various sources, including NADH, FAD, keratin, melanin, and elastin fibers, whereas SHG is used to visualize collagen fibers. It is important to note that these contrast mechanisms rely on the intrinsic optical properties of endogenous tissue biomolecules without using specific fluorescent labels.

In this study, we used an MPM-based clinical tomograph (MPTflex) for imaging of BCC lesions in human skin. This system, as shown in Figure 1, consists of a compact, turn-key femtosecond (fs) laser (MaiTai Ti:Sappire oscillator, sub-100 fs, 80 MHz, tunable 690-1020 nm; Spectra Physics), an articulated arm with near-infrared optics, and beam-scanning module. The imaging head includes 2 photomultiplier tube (PMT) detectors used for parallel acquisition of TPEF and SHG signals. Owing to the high sensitivity of the detectors, the images were acquired in a darkened room. A customized metallic ring taped on the patient’s skin attaches magnetically to the objective holder in the articulated arm, minimizing motion artifacts. The excitation wavelength used for this study was 790 nm. The TPEF signal was detected over the spectral range of 410 to 650 nm, whereas the SHG signal was detected over a narrow spectral bandwidth (385-405 nm) through emission filters placed in the TPEF and SHG detection channels, respectively. We used a Zeiss objective (40×, 1.3 numerical aperture, oil immersion) for focusing into the tissue. The laser power used was 5 mW at the surface of the skin and up to 30 mW in deeper skin layers. The articulated arm of the clinical tomograph allows imaging of almost any part of the body. However, some areas in proximity of the eyes, nose, or ears are still difficult to image.

Study Design
We acquired the MPM images using 2 scanning modalities: The first modality was x-y scanning, resulting in z-stacks of horizontal images from the stratum corneum to the dermis. The z-stacks were obtained by moving the objective in the z-direction, thus scanning at different depths in the skin. The field of view for each optical section was about 200 × 200 μm². Typically, we set a step of 5 μm between the optical sections. Because the optical section is limited to a small scan field, the overall investigation of the lesion required the acquisition of several image stacks of different skin sites. We acquired about 3 image stacks for each lesion. The second modality was x-z scanning, resulting in cross-sectional, “histology-like” images from the stratum corneum to superficial dermis.

The x-y sections were 512 × 512-pixel images acquired at approximately 6 seconds per frame. The x-z sections were 1024 × 1024-pixel images acquired at approximately 30 seconds per frame. Owing to time constraints, we did not acquire x-z sections for all the lesions. This did not influence the results, however, because, the MPM features were clearly identified in the horizontal sections. For the same reason, we did not acquire images of normal skin adjacent to the lesion. Certainly, this would be necessary if the lesion margins are assessed, but this was not the purpose of our study.

Multiphoton microscopy imaging was performed in vivo prior to biopsy. All lesions suspected to be BCC through clinical dermatologic assessment were biopsied and diagnosed using standard hematoxylin-eosin histopathologic protocol.

Results
Nine patients with a total of 10 BCCs underwent imaging shortly before excisional surgery. The histopathologic features of these lesions are listed in the Table and included superficial, nodular, and micronodular BCCs. The main histopathologic findings were nests of basaloid cells extending along the dermoepidermal junction into the papillary dermis (superficial BCC) and various-sized nests invading the reticular dermis (nodular and micronodular BCC). The main MPM features associated with these lesions included (1) nests of basaloid cells present in the papillary and upper reticular dermis, some of which showed palisading in the peripheral cell layer; (2) elongated tumor cells in epidermis aligned in 1 direction; and (3) parallel collagen and elastin bundles surrounding the tumors. A summary of the MPM features found in each lesion is presented in the Table. The morphologic MPM features and the routine histologic findings corresponding to 3 of the 10 lesions are illustrated in Figures 2, 3, and 4. In addition, MPM images and histologic findings corresponding to a micronodular BCC lesion are presented in the eFigure in the Supplement.
Representative MPM images corresponding to 1 of the lesions are shown in Figure 2. A nest of basaloid cells can be seen in both horizontal sections (x-y scan) and the corresponding cross-sectional (x-z scan) images. Particularly in this lesion, the mucinous stroma adjacent to tumor was visualized with MPM. In the standard hematoxylin-eosin-stained histologic appearance, the stroma frequently shows retraction from the tumor islands because mucin shrinks during the process of fixation and dehydration of the specimen, so that the tumor islands appear partially or completely detached from the surrounding dermis. The arrowheads in the images of Figure 2 indicate the mucin adjacent to tumor in the MPM images and its appearance as retraction from tumor islands in the histologic section. Histologic examination revealed palisading of peripheral layers in some of the tumor nests of the lesion, although this feature was not obvious in the nests imaged with MPM.

Representative MPM images acquired in a second lesion are presented in Figure 3 along with the corresponding histologic examination. Both the MPM and the histologic images show a mucinous stroma adjacent to the tumor mass (case 2). B-D, Arrowheads indicate mucinous stroma.
logic section. The MPM images of this lesion show tumor elongated cells aligned in 1 direction (Figure 3B). Palisading of the peripheral layer was present in the MPM images of the BCC nests of this lesion (Figure 3D).

Figure 4 shows the MPM images and the routine histologic findings corresponding to another lesion. The MPM images show the third feature we observed in this study: parallel bundles of collagen surrounding tumor masses, along with palisading of the peripheral cell layer, and tumor cells aligned in 1 direction. The bright cells in Figure 4D and E are most likely melanophages.

Discussion
In this study, we evaluated the potential of MPM to be used as a noninvasive in vivo imaging tool for the immediate and rapid diagnosis of BCC.

The main feature identified in all lesions by MPM imaging was the presence of nested groups of basaloid cells in the papillary and upper reticular dermis. Some of these nests showed palisading at the peripheral cell layer. This feature correlated well with the standard hematoxylin-eosin–stained histologic appearance of near-matching locations. Basal cell carcinoma tumor nests have been previously imaged with various optical imaging techniques.3-7,9,13-18 The morphologic structures inside the tumor nests were clearly observed only in 2 reported studies13,18 on ex vivo imaging of BCC in human skin using FLIM. Our study shows that in vivo MPM imaging can resolve the cellular structure inside the tumor nests corresponding to BCC lesions that include superficial components. This is important because the presence of nests of basaloid cells at the dermoepidermal junction and/or in the dermis is the main histopathologic criterion for BCC diagnosis.20 Therefore, this feature can be potentially used as a criterion for BCC diagnosis with MPM imaging. It still remains challenging to resolve the cellular structure of deeper nests, such as ones found in micronodular BCC (eFigure in the Supplement), but this needs to be investigated in an extended study to include a larger number of nodular and micronodular BCC lesions.

Elongated cells, oriented in 1 direction in the epidermis, have been previously identified in BCC lesions by in vivo RCM8,10,11,13,14 and ex vivo MPM imaging, 13,16 although in the latter study16 this was present in only 1 of 6 tumors. This feature has traditionally been considered an RCM criterion for in vivo diagnosis of BCC because it was identified with high sensitivity and specificity in these lesions.10 While in some studies the elongated cells, oriented in 1 direction are regarded as BCC tumoral cells8,11 or BCC tumoral palisading,10 in others, they were referred to as keratinocytes and most likely regarded as epidermal response to underlying BCC.13,16 In this study, we imaged elongated cells oriented in 1 direction in 4 of 10 lesions. All these lesions were superficial BCCs or nodular BCCs with superficial components. We believe that the presence of elongated cells oriented in a single direction can be explained as appearing only in those BCCs that are superficial enough to be imaged in the epidermis and not, as previously stated,13,16 to be a specific (pathognomonic) feature present in the overlying epidermis of underlying BCCs. If present, elongated cells are related to the palisading of the peripheral cell layer in the intraepidermal BCC nests of basaloid cells. Nevertheless, in 1 of the 4 lesions, the elongated cells oriented in a single direction were present in very superficial layers of the epidermis (Figure 3B). The standard hematoxylin-eosin–stained histologic appearance corresponding to this lesion did not show the presence of this feature in the upper epidermal layers. However, the sampling of heterogeneous tumor will inevitably be somewhat imprecise when attempting to correlate with standard histologic appearance. Horizontal rather
than vertical hematoxylin-eosin-stained sections might be more effective in correlating this phenomenon provided by MPM imaging with histologic appearance.

It is well established that islands of tumor are surrounded by a proliferating connective tissue stroma, which is arranged in parallel bundles surrounding the tumor masses so that a relationship seems to exist between the tumor and its stroma.\(^2\)\(^3\)\(^4\) We found this interesting feature in 5 of the 10 BCC lesions imaged. Parallel bundles of collagen and elastin around the tumor masses have been previously noted in an RCM imaging study of BCC lesions.\(^5\) In a previous study,\(^2\)^\(^4\) the tumor-induced stroma of BCC was associated with the superficial subtype of this tumor. However, in our study, this feature did not indicate a predilection for a particular subtype. Stroma morphologic characteristics of BCC lesions should be further investigated in future studies with larger number of patients.

In this study, we demonstrate that MPM is capable of imaging in vivo tumor nests in BCC lesions of superficial and nodular types along with other features that may be specific to BCC. The MPM imaging was able to visualize the cellular structure inside the tumor nests of BCC lesions that included superficial components. These results provide the groundwork for a future study with a larger number of patients that would assess MPM imaging sensitivity and specificity for in vivo noninvasive BCC diagnosis. One might foresee 2 major technical limitations in performing such a study. In this early stage of MPM clinical translation, limited field of view (about 250 × 250 \(\mu\)m\(^2\)) and penetration depth (about 200-300 \(\mu\)m) are the main technical challenges. High penetration depth is important for imaging tumor nests that are far from the dermoepidermal junction in the dermis, as is often the case for nodular BCC. One way to increase penetration depth is implementing dispersion compensation to decrease the laser pulse duration,\(^2\)^\(^5\) but the gain would be limited. Nevertheless, MPM imaging of tumor nests in several nodular BCC included in this work shows great promise. Scanning large areas of the lesions is important to avoid false-negative diagnoses because lesions are often nonuniform, presenting focal dysplasia and/or malignant neoplasm. The field of view can be increased by implementing a mosaic feature (acquisition of adjacent field of views) or by redesign of the optical components. This technical limitation is being addressed, and implementation has been initiated in a newly developed instrument.

**Conclusions**

This study demonstrates, in a limited patient population, that noninvasive in vivo MPM imaging can provide label-free contrast that reveals several characteristic features of BCC lesions. Future studies are needed to validate the technique and correlate MPM performance with histopathologic findings.

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