Reflectance Confocal Microscopy – Real-Time *In Vivo* Imaging of Basal and Squamous Cell Carcinomas

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Abstract: Reflectance and confocal microscopy (RCM) is an *in vivo* non-invasive imaging tool that captures horizontal images of the epidermis and superficial dermis at nearly the same resolution of routine histopathology. Due to the overlying superficial scaling, RCM characterization of squamous cell carcinomas (SCCs) and associated keratinizing tumors is difficult to visualize due to the obscure appearance of underlying structures. To date, although an increasing frequency of abnormal RCM features are observed across the spectrum, only a few authors have described the features of SCCs. One recent study suggested a disarranged and atypical honeycomb pattern along with round nucleated cells within the spinous/granular layers and round vessels traversing through dermal papilla as key features of SCC.

Meanwhile several RCM features have been linked to basal cell carcinomas (BCCs) regardless of type including:

- Variable architectural disarray of the epidermis
- A uniform pattern of elongated monomorphic nuclei polarized along the same axis
- Abundant vessels with prominent tortuosity
- Mononuclear inflammatory infiltrate admixed with carcinoid basal cells
- Tightly packed cells in the papillary dermis with a nodular/cord-like growth pattern
- Palisading tumor cell nuclei
- Peri-tumoral dark cleft-like spaces representing mucinous edema
- The presence of bright dendritic cells and melanophages in pigmented BCC

Our objective is to identify and describe characteristic RCM findings of SCCs and BCCs by imaging biopsy-proven lesions and reviewing the most recent literature. We will also explain how these features may facilitate diagnosis and recognize future trends for research. Applications of RCM criteria concerning surgical management of these lesions will also be discussed.

Keywords: Reflectance confocal microscopy, Basal cell carcinomas, Squamous cell carcinomas, Actinic keratosis, *In vivo*, Non-melanoma skin cancer, Imaging.

INTRODUCTION

Reflectance confocal microscopy (RCM) is an exciting non-invasive technology that has been developed and is currently being investigated as an adjunct tool to the clinical examination. In reflectance confocal microscopy (RCM), near-infrared light emitted from a diode laser focuses on a microscopic target in the skin. Light is naturally reflected as it passes between different cell structures with different indices of refraction. This reflected light is captured and transferred to compose a two-dimensional gray scale image *via* computer software.

The first investigations using these microscopes attempted to identify the appearance of various cell populations living in different layers of normal skin, such as melanocytes and keratinocytes (Figure **1**). At present, there are a select few number of centers studying squamous cell carcinomas (SCCs) and basal cell carcinomas (BCCs) using RCM imaging of biopsy-proven lesions. In this article, we cover various findings and applications of confocal microscopy to the diagnosis, evaluation, and surgical management of cutaneous neoplasms, particularly BCCs and SCCs. We will also present RCM images with their associated histopathologic and schematic appearance.

SQUAMOUS CELL CARCINOMAS IN RCM

Squamous cell carcinomas are malignant tumors of keratinocytes that arise in the epidermis and are the

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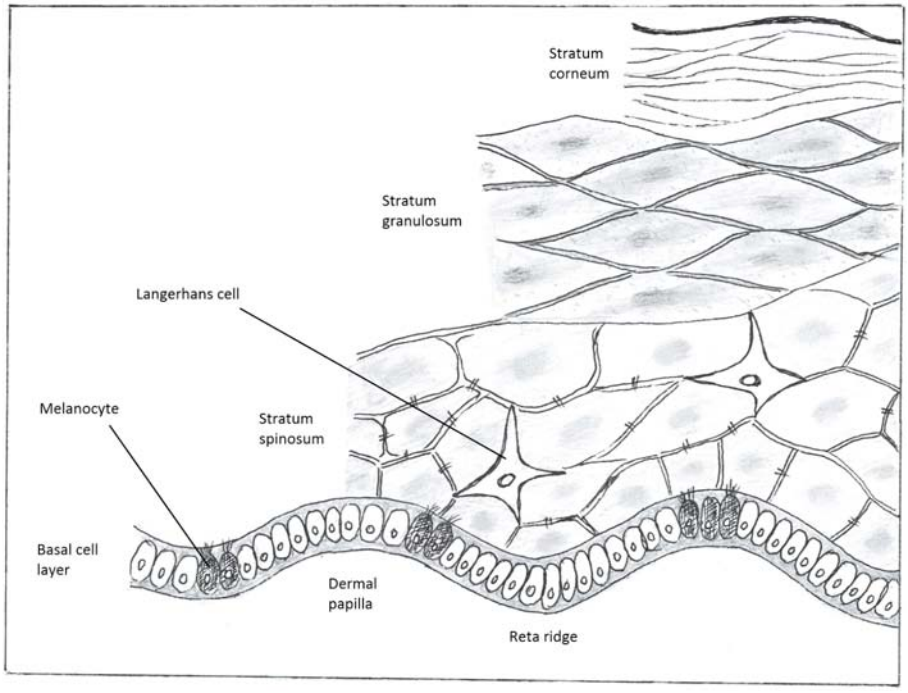

Figure 1: Normal skin – schematic.

second most common skin cancer. SCCs may arise in epidermal precancerous lesions, such as actinic keratoses (AKs), arsenical keratosis, Bowen disease and other epithelial dysplastic lesions involving the epidermis. To date, although an increasing frequency of abnormal RCM features are observed through clinical experience, only a few authors have described the features of SCCs. An initial preliminary report of one case demonstrated significant hyperkeratosis that hindered epidermal imaging at all depths [1].

Regarding the differential diagnosis of keratinized lesions, a distinguishing feature is greater nuclear pleomorphism and architectural disarray in the stratum granulosum of SCCs compared to AKs [2]. A disarranged and atypical honeycomb pattern along with round nucleated cells within the spinous/granular layers and round nucleated cells at the spinous-granular layer are key features of SCC [3]. In 2007, an investigation by Horn and colleagues to validate the diagnostic confocal examination of invasive SCC in microscopy-guided surgery evaluated 120 confocal images of fresh excisions from SCC or normal skin [4]. Regarding the identification of SCCs, general morphology including the location, size and shape of the cancerous lesion could be visualized and densely packed and irregularly organized nuclei and atypia could be delineated, achieving a sensitivity of 95% and specificity of 96.25%, respectively [4]. These sets of well-described morphologic criteria have a powerful diagnostic impact that may be applicable as a rapid diagnostic tool for further investigations and large-scale studies.

SCCs have distinctive vascular features compared to non-melanoma skin cancer (NMSC) and the increased blood flow can be readily observed in real-time video-mode RCM. In two cases of SCC in situ, RCM demonstrated vessels traversing through the papillae perpendicular to the horizontal confocal plane, appearing round in cross section [5]. The vascular supply also appeared exaggerated in number, density, and vasodilation of the capillary loops that normally traverse the dermal papillae [5]. The vascular pattern for SCC in situ was different than for BCCs, appearing perpendicular to the horizontal confocal plane rather than parallel [5].

The diagnostic importance of first analyzing mosaic images at different anatomic levels of the skin is emphasized before focusing on individual high-power images. Increasingly frequent abnormal RCM features can be observed across the spectrum of keratinocyte neoplasias including AKs, SCC in situ, and SCCs (Table **1**). In a clinical series of bedside evaluations of patients with solitary pink lesions, RCM examination allowed for a rapid, comprehensive differential diagnosis and corresponding diagnostic criteria was formulated as follows [6]. RCM mosaic imaging criteria for an AK/SCC include the following: scale and an atypical honeycomb pattern in the stratum

corneum/granulosum/spinosum; superficial disruption with detached corneocytes, large polygonal nucleated cells, and individual highly refractile round cells (parakeratosis) in the stratum corneum (Figure **2**) [6]. Additional criteria include an atypical honeycomb pattern resulting from pleomorphism in size and brightness of keratinocytes and keratinocyte nuclear atypia (large and pleomorphic nuclei) in the stratum granulosum/spinosum; and an inflammatory infiltrate composed of small bright cells in the dermis [6].

Table 1: Characteristic features of BCCs with Reflectance Confocal Microscopy

Variable architectural disarray of the epidermis
A uniform pattern of elongated monomorphic nuclei polarized along the same axis
Abundant vessels with prominent tortuosity
Mononuclear inflammatory infiltrate admixed with carcinoid basal cells
Tightly packed cells in the papillary dermis with a nodular/cord-like growth pattern
Palisading tumor cell nuclei
Peri-tumoral dark cleft-like spaces representing mucinous edema
The presence of bright dendritic cells and melanophages in pigmented BCCs

The combination of RCM and conventional histopathology during Mohs micrographic surgery (MMS) can allow tumor delineation and detection of cancer margins from healthy tissue. In a study on 115 Stage I Mohs surgery excisions including 23 SCCs imaged by RCM and acetowhitening, SCCs were not detected easily due to the bright appearance of the epidermis and lack of cellular detail in the imagery [7]. However, diagnostic aids that characterize SCCs include densely packed atypical keratinocytes and tumor-associated lymphocytic infiltrates [7]. In a study combining RCM with multispectral polarized light imaging for demarcation of NMSCs, thick skin excisions with NMSCs were stained with either toluidine or methylene blue dyes, rinsed in acetic acid, and imaged with the two techniques [8]. Results indicated that CM images correlated with histopathology in hematoxylin & eosin (H&E) staining in terms of the cytological features [8]. There was similarity of the morphological appearance of the tumor microstructure regarding the location and shape of tumor nodules [8]. In a similar study to investigate the feasibility of RCM imaging of shave biopsy wounds using aluminum chloride as a contrast agent to brighten nuclei, atypical cobblestone or honeycomb patterns were identified at the epidermal margins in SCCs, correlating with a proliferation of atypical keratinocytes extending to biopsy margins [9]. RCM of shave biopsy wounds of NMSCs is not only a feasible, complementary alternative, but it also demonstrates a possible technique for intraoperative mapping of surgical wounds in addition to routine frozen section.

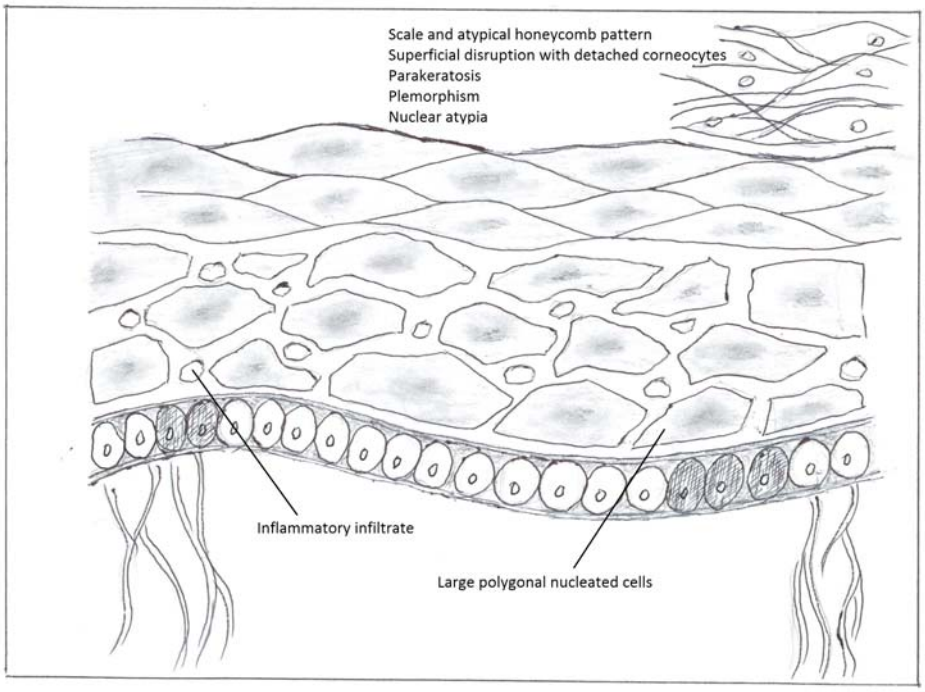

Figure 2: Squamous cell carcinoma – schematic. Keratinocytes appear as large polygonal cells.

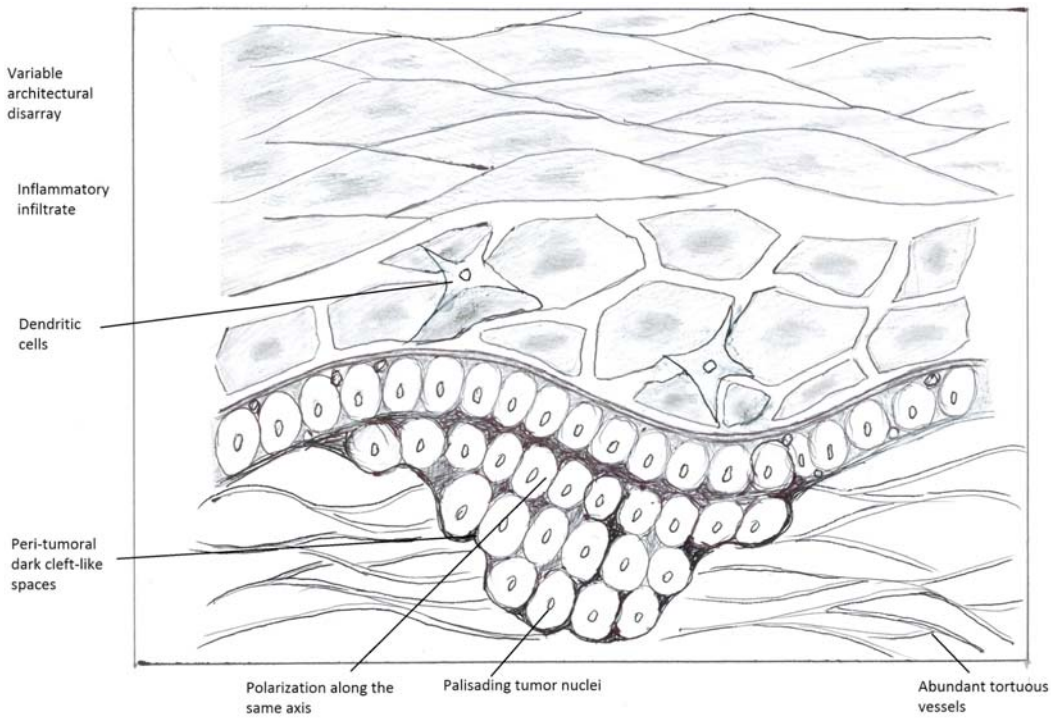

Figure 3: Basal cell carcinoma – schematic. Basal cells correspond to palisading aggregates of round cells and tumor islands.

BASAL CELL CARCINOMAS IN RCM

Basal cell carcinoma is the most common cancer in humans that can be clinically characterized into different types including: nodular, ulcerating, pigmented, sclerosing and superficial. In 2004, Nori and colleagues explored the sensitivity and specificity of RCM for diagnosis of BCCs in 152 lesions using five previously described criteria (prominent inflammatory infiltrate; increased vasculature; polarized nuclei; elongated nuclei; and pleomorphism of the overlying epidermis indicative of actinic damage) in a blinded study (Figure **3**, Table **2**) [10]. The most relevant feature was the presence of polarized nuclei along the same axis of orientation and the presence of elongated monomorphic basaloid nuclei was the most sensitive criterion for diagnosis at 100% sensitivity [10]. Results indicated that 4 or more of the RCM criteria presented a specificity of 95.7% and two or more criteria were 100% sensitive for the diagnosis of BCC [10]. In an evaluation of the diagnostic accuracy of 12 fresh BCC excisions by RCM, most of the features were highly reliable including tumor cell nuclei and tumor nests [11]. In contradistinction, disintegration of tumor cells, peripheral palisading, and refraction of stroma was hardly useful [11]. The promising results from this study opens avenues for future studies in which RCM can serve as a guide for microsurgery of any type of skin cancer.

Table 2: Characteristic features of SCCs with Reflectance Confocal Microscopy

Scale and atypical honeycomb pattern
Superficial disruption with detached corneocytes
Large polygonal nucleated cells
Individual highly refractile round cells (parakeratosis) in the stratum corneum
Pleomorphism in brightness and size of keratinocytes
Keratinocyte nuclear atypia (large and pleomorphic nuclei)
Inflammatory infiltrate composed of small bright cells

Pigmented BCCs often represent a diagnostic dilemma and may be indistinguishable from superficial spreading or nodular melanoma. Several authors have affirmed the presence bright dendritic structures with small plump cell bodies and branching processes, identified histologically as either melanocytes or Langerhans cells, consistently seen within the epidermis overlying the tumor or within tumor islands in pigmented BCCs [12, 13]. In 2006, Agero *et al*. imaged pigmented skin lesions with a differential diagnosis of pigmented BCC using dermoscopy and RCM followed by excision for histologic analysis [12]. RCM showed aggregates of well-circumscribed tightly packed palisading cells forming trabeculae/cordlike structures and polypoid islands or nodules with border irregularity and variable brightness which represented nests of pigmented basaloid tumors cells on histology and blue-

gray ovoid areas on dermoscopy [12]. The tumor nests were associated with scattered, bright oval, plump to stellate structures with indistinct borders, representing melanophages with highly refractile melanin granules [12]. In a study of three consecutive pigmented BCCs by RCM alongside histological and immunohistochemical correlation, highly refractive dendritic structures located within tumor nests correlated with the presence of melanocytes within the tumor by immunohistochemical analysis [14]. Although RCM is unable to differentiate melanocytic dendritic cells from Langerhans' cells, immunostaining show the predominance of Langerhans' cells in the epidermis and melanocytes within the tumor islands [14]. Thus, in pigmented BCCs, dendritic melanocytes can be easily identified by RCM.

Distinguishing the vascular features of BCCs using RCM may also serve as a useful diagnostic tool. Vessels are "canalicular" like funneling passageways and have been described as branched, linear structures that run parallel to the horizontal plane of RCM imaging [15]. The vessels branch and intertwine between tumor aggregates, an abnormal pattern apparent on RCM as well as dermoscopy [15]. This pattern likely reflects a known feature of carcinogenesis, neoangiogenesis. Its difference from the vascularity of SCCs and seborrheic keratosis can also serve as a distinguishing feature in the differential diagnosis of NMSCs and other cutaneous lesions. In a study of 12 patients with a histologic diagnosis of BCC, the images on RCM were compared to histological examinations of excised tissue [16]. In all BCCs, typical changes in vascularity such as loss of architecture, increased number and diameter of vessels, parallel and horizontally-oriented vessels, and accumulation and rolling phenomena of bright reflecting cells along the vessel wall were observed [16]. The stroma demonstrated strong reflectance due to dense collagen bundles encasing dark, cell-rich silhouettes of tumor parenchymal cells [16]. Five patients had narrow basaloid cells with polarization of large, elongated dark nuclei at the periphery [16]. In fibrosing BCCs, curled collagen bundles with large cells represented the stroma component of the tumor. Thus, these changes in the vasculature can serve as criteria for BCC and can help diagnose BCC by RCM and assess the margins of large tumors in the future prior to surgical intervention.

There is excellent correlation between *in vivo* confocal imaging and standard microscopy of H&E stained tissue sections that may facilitate *in vivo* diagnosis of BCC with high-resolution criteria. In a

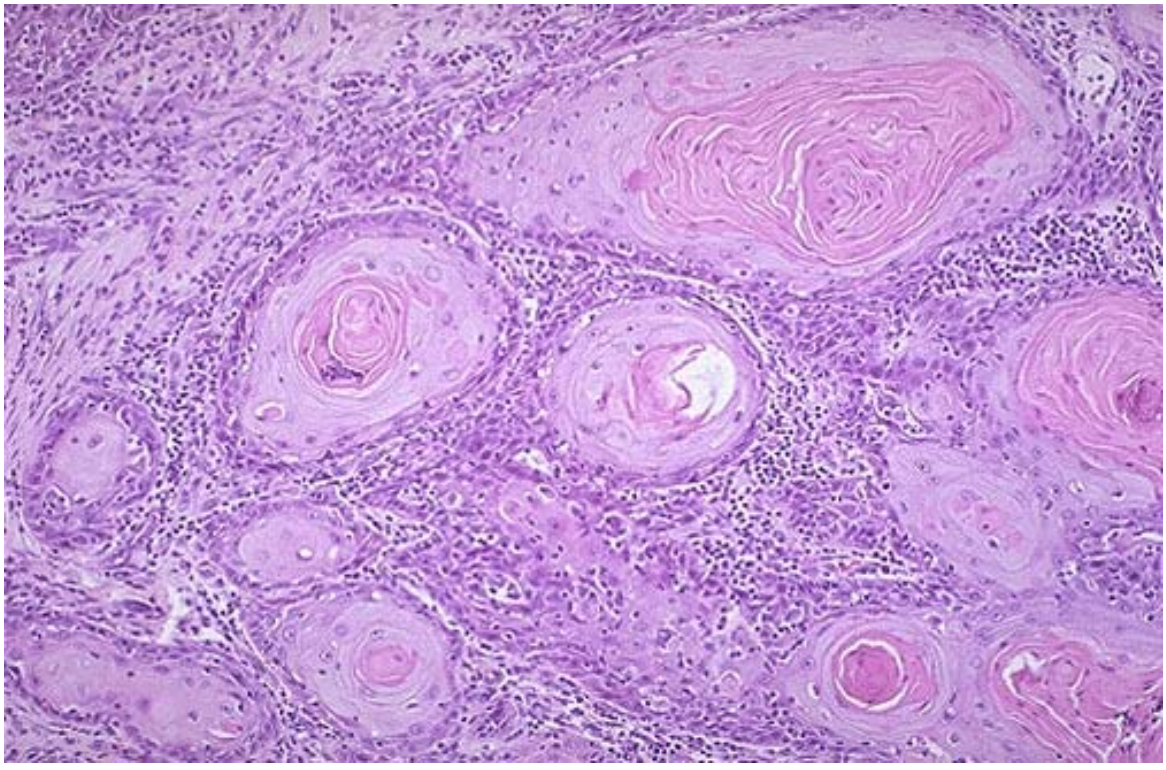

Figure 4: Squamous cell carcinoma – histopathology. Histopathology shows keratinocyte atypia and islands of invasive SCC with peritumoral inflammation, increased vascularity, and keratin pearls.

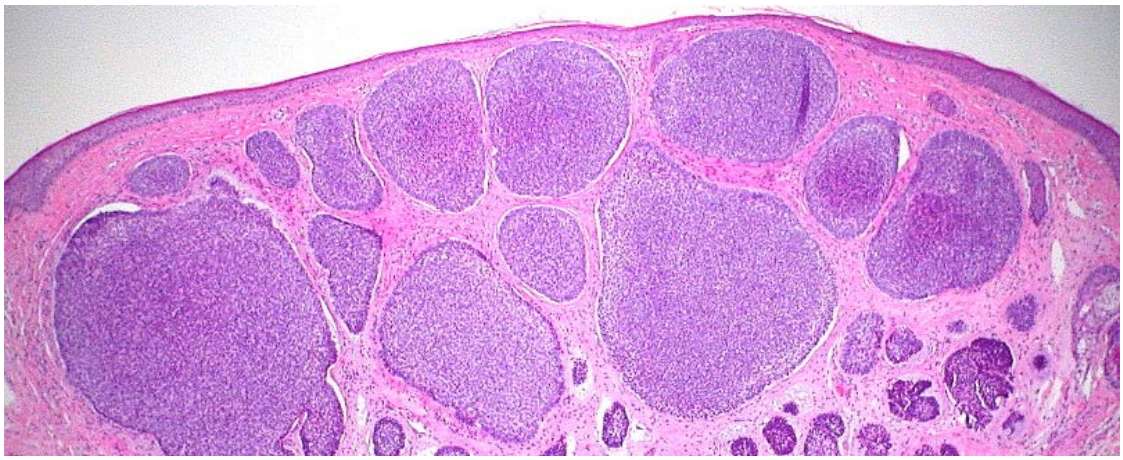

Figure 5: Basal cell carcinoma – histopathology. Histopathology shows dermal islands of atypical basaloid cells with scant basophilic cytoplasm and large ovoid nuclei. Focal peripheral palisading of nuclei is surrounded by cellular fibrous stroma.

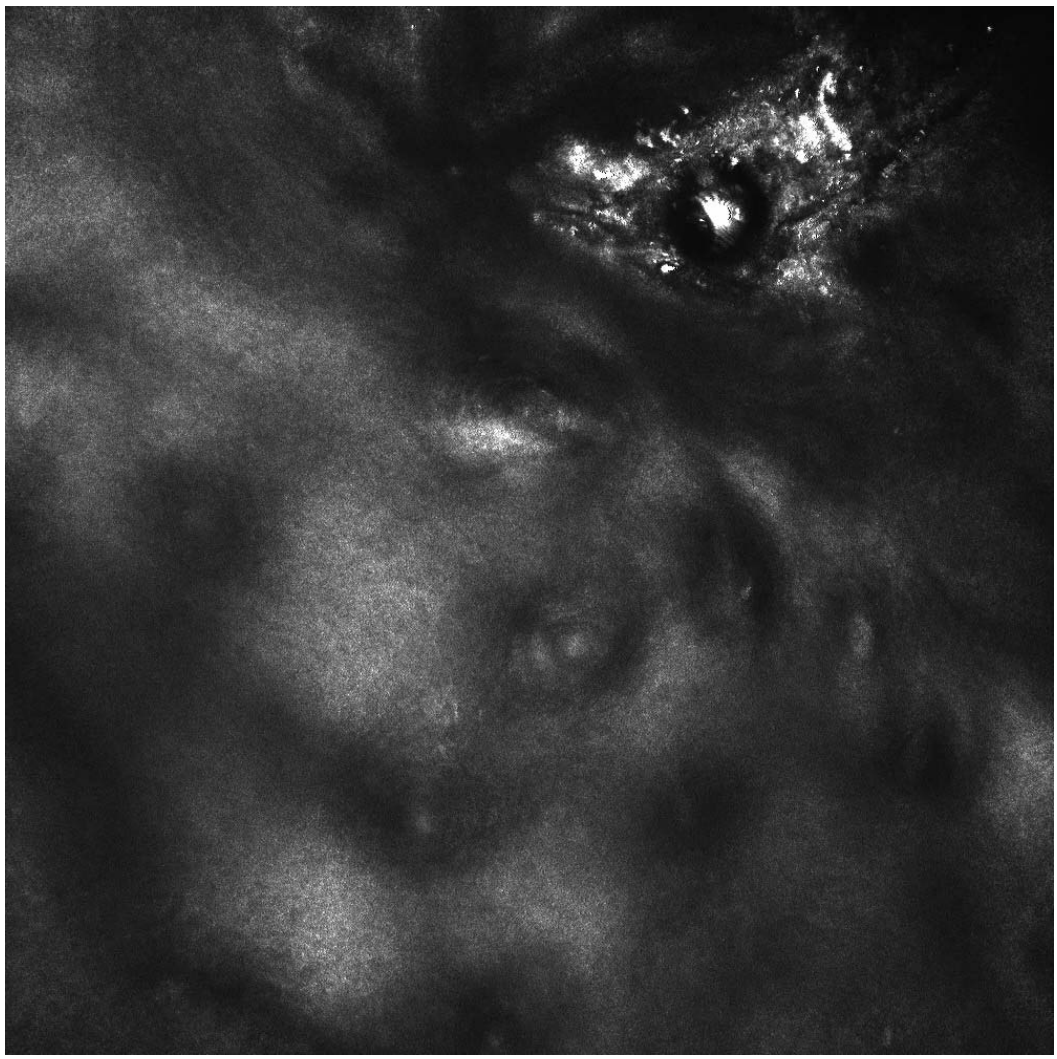

Figure 6: Squamous cell carcinoma – RCM. There are rounded granular highly refractile cells at the level of the stratum corneum suggestive of scale. The upper epidermis reveals severe disruption of the keratinocyte architecture, resulting in an atypical honeycomb pattern.

study by Gonzalez and Tannous to define the *in vivo* histologic features of BCC by RCM, eight BCCs were studied by RCM and the features were correlated with H&E staining obtained from the corresponding biopsies

[17]. A uniform pattern of elongated monomorphic nuclei polarized along the same axis was always present and trafficking of leukocytes was also visualized [17]. Abundant vessels with prominent tortuosity and a prominent mononuclear inflammatory infiltrate admixed with carcinoid basal cells were observed [17]. In a pilot study detecting residual or clinically equivocal BCCs using RCM, characteristic features including tightly packed nests of elongated monomorphic polarized nuclei and subjacent ectatic vessels with lymphocytes undergoing rolling and margination were revealed [18]. In these cases, conventional histology with H&E staining obtained during MMS confirmed the presence of BCCs [18]. Similarly, another study using acridine orange in fluorescence and acetic acid as a contrast agent was shown to enhance nuclear contrast and enable detection of residual BCCs with high accuracy at approximately 96.6% and 89.2% sensitivity and specificity, respectively [19]. Though RCM may facilitate diagnosis of BCCs, further studies to evaluate the accuracy of histologic criteria are warranted.

The use of different contrast agents has shown promise in skin surgery and other medical uses. In an evaluation of the feasibility of RCM as a surgical guide in MMS, Tannous *et al*. applied 20% aluminum chloride (AlCl) on the defect followed by RCM on one site from each lesion [20]. AlCl proved to be an excellent contrast agent, highlighting the intensely bright nuclei of tumor cells [20]. Differences between cancerous basaloid cells and the surrounding tissue were readily

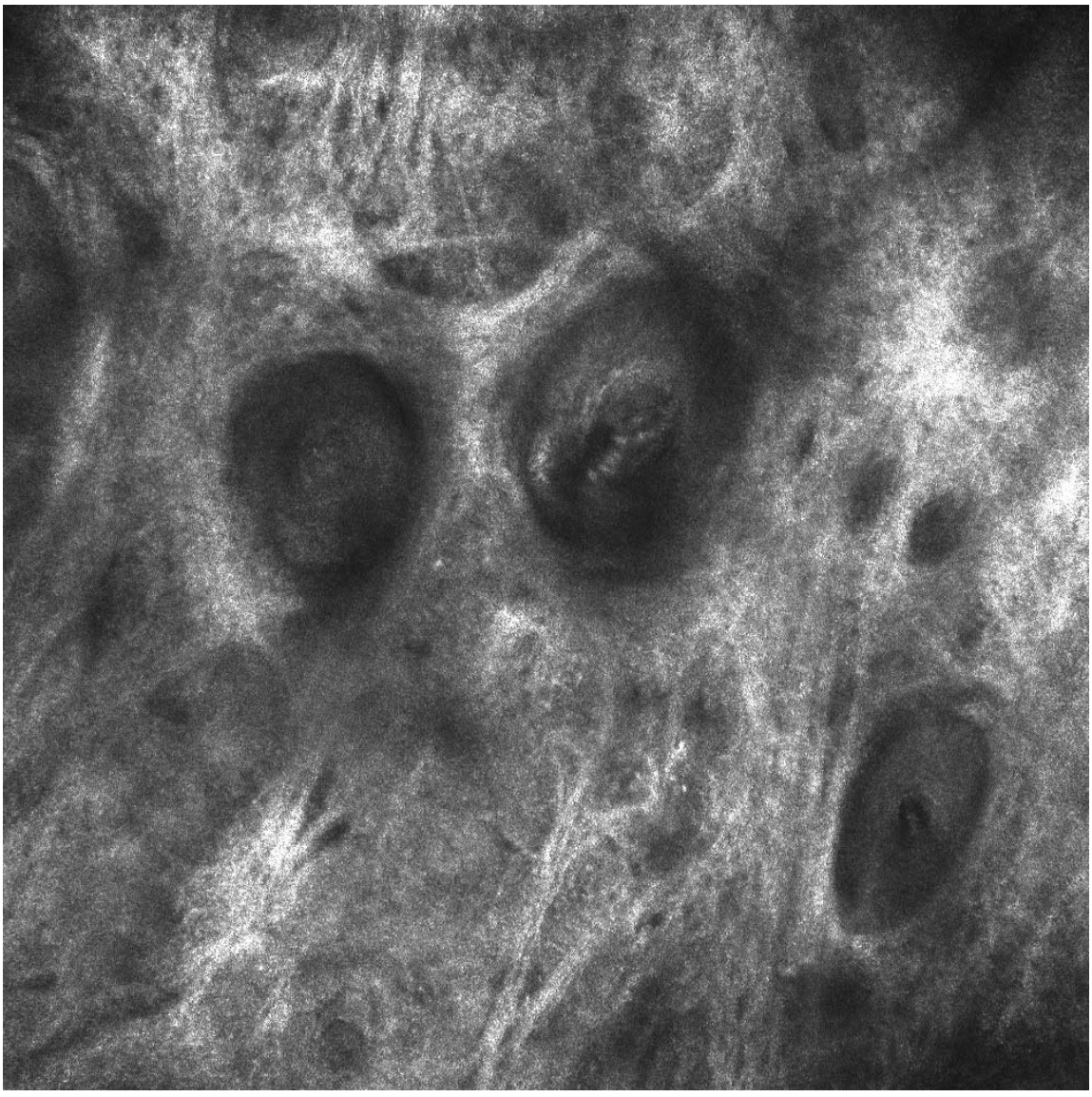

Figure 7: Basal cell carcinoma – RCM. At the level of the dermis there are tumor islands composed of tightly packed, weakly to moderately refractile cells with peripheral palisading nuclei surrounded by moderately refractile stroma and collagen bundles.

detected, suggesting RCM as a potential guide and AlCl as a superior exogenous agent to enhance tumor contrast. In a series of 13 BCCs studied by RCM and histopathologic techniques by Ulrich *et al*., Alcian blue staining was performed to detect peritumoral mucin and the thickness was measured [21]. Results showed that peritumoral cleft-like spaces on histopathology correlated with dark areas on RCM and correspond to the thickness of peritumoral mucin deposition [21]. These findings demonstrate the RCM may facilitate diagnosis of BCC *in vivo* in cases in which biopsies may be inadequate. The use of different contrast agents for image enhancement serve as an important step towards the long-term clinical goal of noninvasive imaging modalities for potential real-time surgical pathology at the bedside of the skin and tissues of other organs.

LIMITATIONS OF REFLECTANCE CONFOCAL MICROSCOPY

The major drawback of RCM as a detection and guidance technique for BCCs and SCCs is the small field of view (approximately 0.3 mm). However it is possible to enlarge the field of view up to 2 mm by reducing the resolution in the axial plane. To thoroughly examine the entire suspicious lesion, a sequence of images must be captured and incorporated together. This sequencing process takes time and motion artifacts or the inability to be still may result in a distorted image. Another practical limitation of RCM is the requirement for a flat, static sample surface; however, this is relatively simple to achieve regarding SCCs and BCCs.

The length of time required to train the eye of a health professional to detect characteristic criteria of BCCs and SCCs may not be feasible to serve as a complementary tool in the intraoperative setting. Due to the overlying superficial scaling, RCM characterization of SCCs and associated keratinizing tumors is difficult to visualize due to the obscure appearance of underlying structures. Regarding SCCs, Chung *et al*. concluded that assessment of nuclear atypia was difficult and individual keratinization features were not visualized due to the inability of the RCM to visualize keratin [7].

Different sets of criteria may be necessary for detection of the different subtypes of infiltrative or atypical BCCs. According to Patel *et al*., comparison of RCM mosaics of excisions soaked in acetic acid to histopathology easily detects nodular, micronodular, and superficial BCCs, however, infiltrative and sclerosing BCCs tend to be obscured within the surrounding bright dermis [22]. Further studies are needed to evaluate the accuracy of different features of characteristic diagnostic criteria that have been proposed thus far.

CONCLUSIONS

Whether RCM can create images with sufficient detail to bypass histological analysis of tissue biopsies is a goal that may not be far from reality. In addition to serving as a guide to surgery, RCM assessments may also help determine response to therapy and the need for surgical intervention. A study by Torres *et al*. to determine if RCM is useful to establish the need for surgery after imiquimod evaluated the efficacy of 5% imiquimod cream 5x/week for two, four or six weeks in treating BCC preceding Mohs excision [23]. RCM assessment correlated well with histologic diagnosis and response to therapy with imiquimod [23]. In a similar study to assess the efficacy of cryotherapy for superficial BCCs by cytomorphologic analysis using RCM, early cell necrosis within upper dermal structures presenting as black round to oval areas of varying size with bright floating structures correlated with ablation of overlying tumor tissue and incipient blistering [24]. When these findings are not reproduced by treatment of the malignant lesion, additional treatment sessions should be considered to fully eradicate the tumor.

In the future, confocal laser-scanning microscopy can serve as a guide for microsurgery of BCCs and SCCs, as well as other skin cancers. Fewer than 25% of graduating U.S. medical students have never observed a skin cancer examination, 26.7% have never been trained to perform one, and 43.4% have never examined a patient for detection of skin cancer [25]. This translates into the fact that several recently graduated physicians could be undertrained in detecting skin cancers. Programs and advancements in technology, such as real-time *in vivo* reflectance confocal microscopy, need to be implemented in order to increase NMSC diagnostic skills. New non-invasive diagnostic techniques such as RCM may lead to enhanced diagnostic accuracy and complementary methods in the near future.

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