The use of reflectance confocal microscopy for monitoring response to therapy of skin malignancies

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Summary

Reflectance confocal microscopy (RCM) is a new non-invasive imaging technique that enables visualizing cells and structures in living skin in real-time with resolution close to that of histological analysis. RCM has been successfully implemented in the assessment of benign and malignant lesions. Most importantly, it also enables monitoring dynamic changes in the skin over time and in response to different therapies, e.g., imiquimod, photodynamic therapy, and others. Given the often traumatic nature of skin cancer that affects both the physiology and the psychology of the patients, it is crucial to have methods that enable monitoring the response to treatment but that minimize the distress and discomfort associated with such process. This article provides a very brief overview of the fundamentals of RCM and then focuses on its recent employment as a monitoring tool in skin cancer and other pathologies that may require frequent follow-up.

1. Introduction

Non-invasive techniques are the necessary future of diagnostic procedures. In dermatology, biopsy collection may be very invasive, producing scarring and malformation of the skin and having a deleterious aesthetic and psychological effect.

As imaging techniques have evolved recently, new possibilities have opened for the dermatology practitioner to gather information on the status of skin and external mucosal tissue in a non-invasive manner. The advantages of these new techniques include an increased precision and delimitation of the margins of a lesion, reduced distress for the patient, and the capability to perform repetitive analysis of the affected area. These techniques include dermoscopy [1], optical coherence tomography [2], high-frequency ultrasound [3], magnetic resonance imaging (MRI) [4], fluorescence-mode confocal microscopy [5], and reflectance-mode confocal microscopy (RCM) (see below). The major advantage of conventional, biopsy-based histology is its diagnostic

value and its resolution power. However, non-invasive imaging is painless; it causes no tissue damage and its resolution is rapidly improving. Also, processing-based artifacts (due to fixation, sectioning, and mounting) are not an issue, since non-invasive imaging does not disrupt the native structure of the tissue. In addition, non-invasive in vivo imaging is less time-consuming (e.g., it obviates sample processing) than routine histology. Finally, the specialist can sample the same location of the skin over and over to collect a series of images. This improves the quality of the data and offers an invaluable advantage when treating diseases. Furthermore, it allows sequential evaluation of therapies that alter the architecture of the skin, including pre-surgical tumor evolution, post-surgical wound healing, evolution in response to non-surgical therapy and determination of outcome (e.g., remission vs. relapse) [6-10]. Two major caveats stand out: one is that the skin is only a semi-translucent tissue, hence, light-based applications meet with physical barriers that stop photons and complicate obtaining quality data from the lower layers of the skin. The second problem is that histology is a well-established technique with over 100 years of accumulated data and evolution in data collection, staining and quantification. In comparison, these non-invasive techniques are relatively young and findings obtained through them still need to be correlated with histology to ensure the correct diagnosis is achieved. This latter caveat is being overcome by recent efforts to endow the literature with a consensus terminology [11] and to publish atlases detailing observations made using these novel techniques and correlating them with conventional histology, e.g., the handbook of the use of RCM in dermatology [12]. These efforts offer multiple beneficial effects for the dermatology community: 1) they provide informative tools for dermatologists and imaging technicians; 2) they homogenize the nomenclature, which is an essential, often overlooked step for these techniques to gain widespread acceptance; and 3) they often help in clarifying diagnosis by correlating the findings in the clinic with published case reports.

This review will focus on the employment of RCM in monitoring response to non-surgical skin treatments.

2. Principles and technology of reflectance confocal microscopy

Confocal microscopy is a relatively common tool used in basic sciences (cell biology, immunology or neurobiology), but its in vivo application has required major improvement in optics, light sources and system stabilization for the images to have enough quality to become useful. In 1995, confocal scanning laser microscopy was first reported to image human skin in vivo [13]. This opened the gates to its employment in a varied array of skin disorders and diseases.

Reflectance confocal microscopy collects the light reflected from specific structures (normally cells) present in small areas of the skin scanned with a low power laser. This approach generates images of dark (non-reflecting) and bright (reflecting) structures in the skin, and represents them as thin sections of horizontal tissue in vivo.

RCM microscopes are, in fact, in vivo adaptations of confocal microscopes used in basic sciences. RCM microscopes use lasers as sources of illumination. The actual range of RCM varies from 800 to 1064 nm; and the lasers used in RCM are not very powerful (<30 mW) to avoid damaging the tissue during examination; the maximum depth of imaging being between 200 and 250 μm [6,14]. Another important component of RCM is the skin contact device, which is used to minimize spherical aberrations and movement artifacts. This is done through the use of a water-gel interface between the lens and the skin in a vacuum type device that encases the lens. A typical example of lens used in RCM is a 30x objective lens of numeric aperture (NA) of 0.9, which, together with a physical filter provides a lateral resolution of approximately 1μm and an axial resolution (section thickness) of 3-5 μm [14]. Finally, RCM allows for the collection of time-lapse photography to visualize dynamic events in the skin, e.g., cell migration or blood flow [6,8,10,13].

3. Applications of reflectance confocal microscopy for dynamic monitoring over time

The best-characterized application of RCM in the dermatologist's office is diagnosis, and a number of reviews have covered the topic recently [15-18]. This article, on the other hand, will focus on the employment of RCM in monitoring response to surgical and non-surgical skin treatments.

The gold standard for the treatment of several types of skin cancer is surgical removal (excisional biopsy) of the affected area and the adjoining healthy tissue to prevent the escape of tumor cells. However, this is often traumatic, particularly if the affected area is a cosmetic hotspot, e.g., the face. Excisional biopsy is often preceded by one or several incisional biopsies aimed at characterizing the tumor, determining malignancy, and helping the medical practitioner make an informed decision on the most appropriate treatment. Furthermore, excisional surgery needs to be followed up as well and may require additional incisional surgeries. With these problems in mind, the development of non-invasive treatment protocols has been important. Major breakthroughs are the uses of photodynamic therapy (PDT) and imiquimod application.

3.1. Photodynamic therapy
PDT is a minimally invasive therapeutic protocol that induces selective cytotoxicity toward tumor cells. A photo-
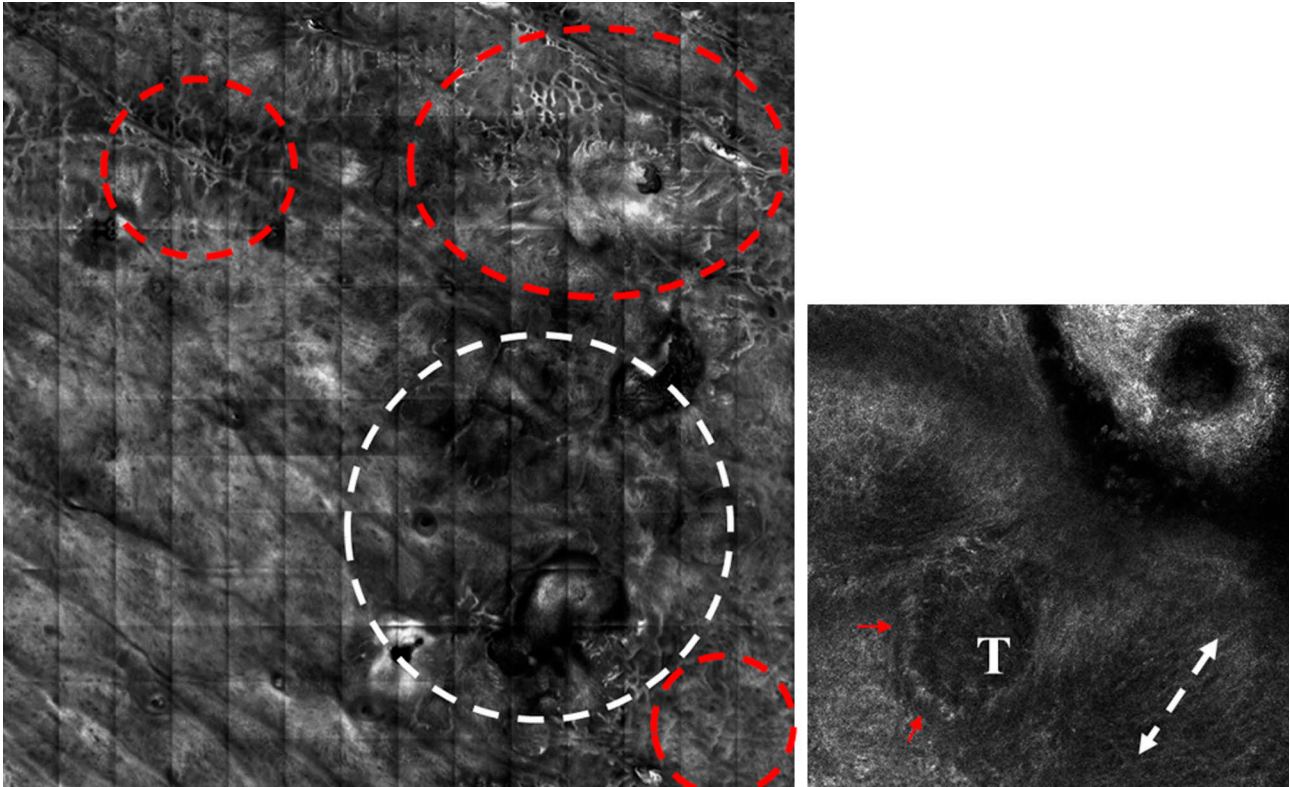
Figure 1. Confocal images from the left shoulder of a 73-year-old female patient. In that area, a lesion was diagnosed of expansive type basal cell carcinoma and treated with MAL (methyl aminolevulinate) PDT. Confocal images A and B were taken when patient presented at the clinic for revision without clinical and dermoscopic evidence of recurrence. Figure 1(a) shows an RCM mosaic (7 mm × 7 mm) at the level of the upper dermis revealing disarranged area (white dashed circle) surrounded by lentiginous background (red dashed circles, large, polymorphic and numerous dermal papillae). Figure 1(b) shows an RCM image (0.5 mm × 0.5 mm) at the level of the upper dermis revealing a lobulated tumor island (T) with subtle peripheral palisading of nuclei (red arrow). A population of basaloid cells with characteristically elongated nuclei oriented along the same axis (dashed white line) forming typical "nuclear polarization" is seen. [Copyright: ©2012 Ulrich et al.]

sensitizing agent is applied and kept under occlusion for several hours, followed by irradiation at the appropriate wavelength to activate the sensitizer. In the presence of oxygen, generation of local reactive oxygen species (ROS) leads to direct tumor cell apoptosis, necrosis and autophagy, and to induction of a local inflammatory response. Furthermore, the effect of PDT on tumor clearance is potentiated by vascular damage to the vessels in the vicinity of the tumor, which limits oxygen supply. The most common sensitizing agents are porphyrin derivatives, especially 5-ALA (5-aminolevulinic acid) or its methyl-ester, methyl-aminolevulinic acid (MAL, commercialized under the name Metvix®), which are excited at ~635 nm. PDT using MAL has been utilized in a number of skin cancers, e.g., basal cell carcinoma (BCC), to either treat the tumor directly or to reduce the size of the lesion prior to surgery.

3.1.1. RCM monitoring of the response of BCC to PDT treatment (Figure 1)

PDT treatment has been very successful in clinical trials (reviewed in [19]). Different histological subtypes of BCC have been treated with PDT. For example, treatment of nodular BCC entails a higher risk of relapse than conventional surgical excision [20]. Conversely, PDT of superficial BCC has an efficacy comparable to that of surgery, but with a cosmetic outcome that is vastly improved [21]. Several studies have addressed the potential of RCM to follow up the response of BCC lesions to PDT using MAL [22]. Gorlin syndrome (GS) and xeroderma pigmentosum (XP) are congenital skin diseases that share a genetic predisposition to develop skin cancer. Patients suffering from these genodermatoses often develop BCC. One recent study used MAL-based (Metvix®) PDT in a small group of GS and XP patients and followed up tumor cell clearance by RCM. Single or multiple lesions in localized areas were treated with 1–3 cycles of MAL PDT. Every cycle was comprised of two sessions of PDT seven days apart. Patients were evaluated after three months and followed thereafter every six months for three years. Under these conditions, this study reported excellent results in the use of RCM to determine the degree of clearance after PDT [22].

3.1.2. Treatment of actinic keratosis with PDT in solid-organ transplanted patients: RCM follow-up

5-ALA PDT is rapidly becoming a reference treatment in the management of actinic keratosis (AK) [23]. In addition to close to 100% efficacy, the cosmetic outcome of the procedure

is vastly superior to that of surgery. An area of particular interest is the management of skin cancer, mainly SCC, of which AK is considered a direct precursor [24] in immunosuppressed individuals, e.g., those undergoing solid organ transplantation. In this context, a recent study has evaluated, using RCM, the response to 5-ALA PDT in organ transplant recipients (OTR) that display AK. In this study, RCM proved to be an excellent tool to monitor the response to PDT [25]. This application of RCM is particularly important for immunocompromised individuals who have a high prevalence of AK.

3.2. Imiquimod treatment

Imiquimod (3-(2-methylpropyl)-3,5,8-triazatricyclo[7.4.0.0^{2,6}] trideca-1(9),2(6),4,7,10,12-hexaen-7-amine) is an immunomodulator that promotes antitumor immunity driven by dendritic cell (DC) and macrophage recruitment to the tumor area and production of cytokines [26]. These cells then take up apoptotic and necrotic tumor cell bodies. Tumor antigen-loaded DCs then migrate into the lymph nodes and promote T-cell mediated tumor immunity [27]. Imiquimod has received FDA-approval for the treatment of actinic keratosis, superficial basal cell carcinoma, and external genital warts, and is commercially available under various trade names, e.g., Aldara®, Zyclara®, Beselna®, or R-837.

3.2.1. RCM monitoring of the response of BCC to imiquimod (Figures 2-4)

In 2003, our group reported the use of RCM for the diagnosis of BCC and for monitoring BCC clearance following the application of imiquimod [28]. The patient underwent a three-week course of daily application of a thin layer of 5% imiquimod cream to the clinically involved area. RCM demonstrated in exquisite detail the week-to-week response of the affected area to the treatment. RCM revealed intense inflammation, but after three weeks, there were no clinical signs of BCC. In 2004, another study used RCM to determine the effect of imiquimod treatment on BCC prior to Mohs microsurgery [29]. The area of the lesion was treated five times/week for two, four or six weeks, then Mohs microsurgery was performed two to four weeks after completion of imiquimod treatment. RCM was used to assess pre-surgical tumor clearance with an efficacy comparable to that of histology. This study suggests that RCM can be used to monitor the response of a lesion to imiquimod, aiding in the patient's and dermatologist's decision on whether surgery is further needed.

3.2.2. RCM monitoring of the response of actinic keratosis to imiquimod

The applicability of RCM to evaluate the response of AK to imiquimod has been assessed in a small group of patients with subclinical features of AK [30]. In these patients, RCM was used successfully to characterize the subclinical phenotypes of AK before treatment, as well as to assess the effect of imiquimod throughout a four-week course of treatment (which included three applications/week). Another study compared vis-a-vis the effect of imiquimod (three times/week for four weeks) and 5-ALA PDT in AK in organ transplantation patients [25]. This study, which involved imiquimod treatment of AK on the balding heads of patients, reports that RCM allowed excellent visualization of the evolution of response. This study was used to generate a table of RCM-visualized parameters and their usefulness for diagnosis of AK and for monitoring its response to imiquimod or PDT. However, the sample size was small, and larger numbers are required to make these into general guidelines. An additional case of AK with SCC features was treated with a combination of imiquimod, tretinoin, and 5-fluorouracil [31]. The combined regimen was applied once daily for three weeks, followed by a two-week rest period, and then by another three-week treatment cycle. RCM evaluation 8 weeks after completing the treatment revealed improvement, although complete clearance was not achieved.

3.2.3. Treatment of melanoma with imiquimod and monitoring by RCM

Although imiquimod is not the reference treatment for melanoma, it has been assayed for the treatment of amelanotic or poorly melanotic melanoma and lentigo maligna melanoma (LMM). This is particularly important for LMM due to the fact that these tumors are heterogeneous, display poorly defined margins and appear in cosmetically sensitive areas, where surgery has an elevated risk of disfigurement [32]. In general, the response of melanoma to imiquimod has been often positive [33], but some foci seem to be resistant, casting severe doubt on this approach for long-term melanoma management [34]. RCM has been used to monitor the response of melanoma to imiquimod therapy in several studies. In one example, imiquimod (16-week course, one application/day) was used to treat one clinical case of melanoma where advanced age and other clinical complications prevented alternative treatments [31]. In this case, RCM revealed complete clearance after one year. In another case of amelanotic melanoma, the patient used 5% imiquimod cream twice a day for one week until erythema and irritation occurred, and then once a day for four additional weeks. Mid-treatment RCM evaluation revealed inflammation, with clearance of atypical melanocytes. Imiquimod therapy was restarted, with application every three days for six months to minimize significant erythema and irritant response. At the 1-year follow-up visit, RCM examination revealed no evidence of relapse [35]. A recent report describes one case of in situ melanoma (lentigo maligna type) treated over 27 weeks with daily imiquimod, but with significant treatment
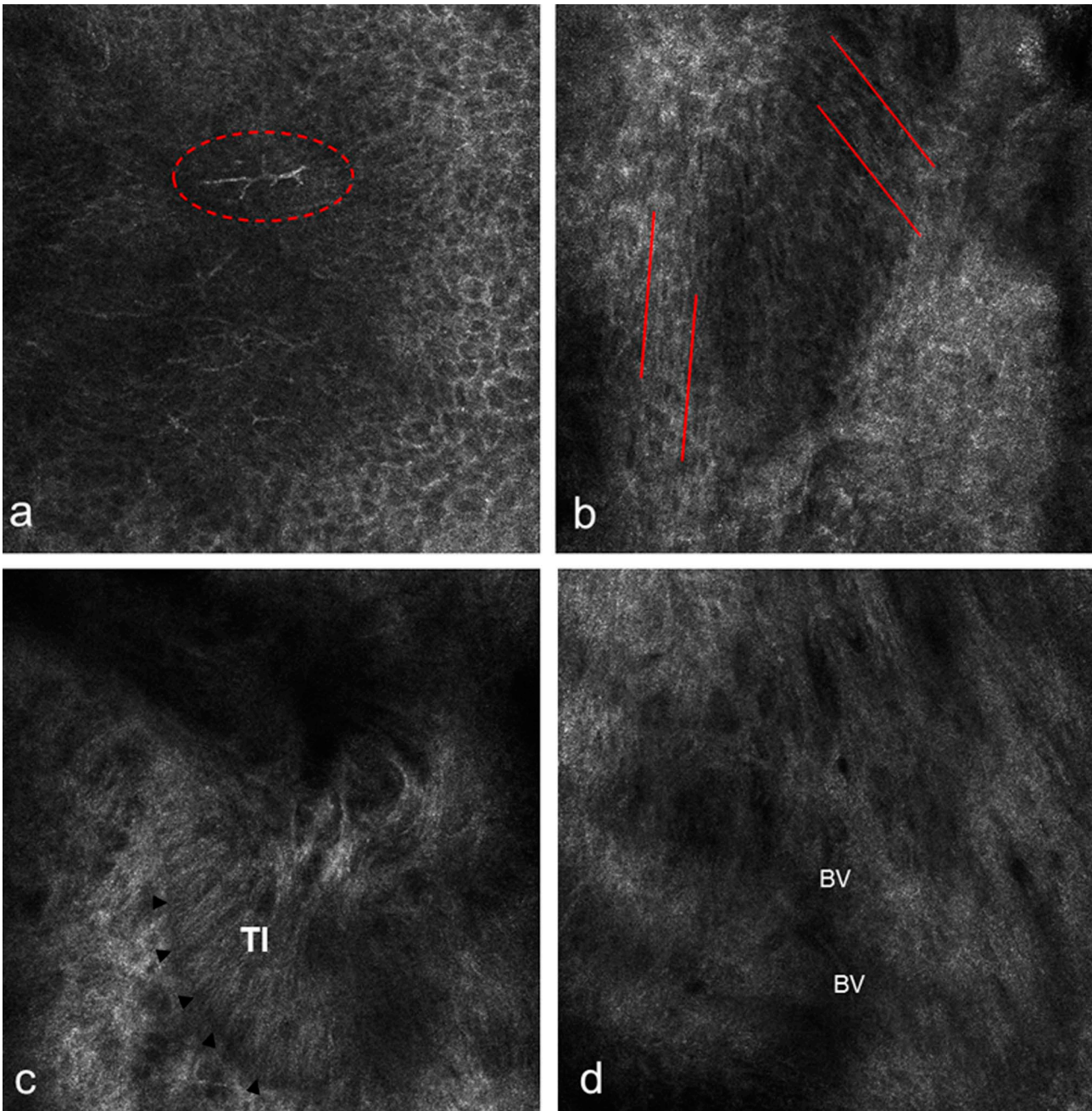
Figure 2. Morphological RCM changes as visualized during treatment of BCC with 5% imiquimod cream. Superficial BCC before initiation of treatment. 1(a) is showing the presence of dendritic cells (red dashed circle) in the epidermis overlying the BCC, likely corresponding to Langerhans cells. 1(b) illustrates polarization of elongated cells at the basal layer of the epidermis (red lines). 1(c) shows the presence of a tumor island (ti) with peripheral palisading of cells and nuclei that are separated from the surrounding fibrous stroma by a dark, cleft-like space (black arrowheads). Figure 1(d) displays dilated blood vessels (BV) in the superficial dermis. [Copyright: ©2012 Ulrich et al.]

breaks due to inflammatory complications. In this case, complete clearance as revealed by RCM was not achieved and surgical excision was necessary [32]. The same study reports another case of in situ melanoma, lentigo maligna type. The patient turned down surgery due to cosmetic complications and, instead, underwent daily treatment with imiquimod for three months. Before and after RCM monitoring revealed complete clearance up to one year after completion of the treatment [32]. A third case of melanoma on sun-damaged skin, treated daily with imiquimod for three months, was

reported. RCM examination three and six months after treatment did not reveal any sign of relapse and instead showed recuperation of the normal morphology of the different epidermal layers [32].

3.3. Cryotherapy and shave biopsy

Recent studies have reported the use of RCM to monitor the response to two other types of therapy procedures: shave biopsy for AK [36] and cryotherapy for BCC [37]. In the AK study, the authors performed shave surgery in
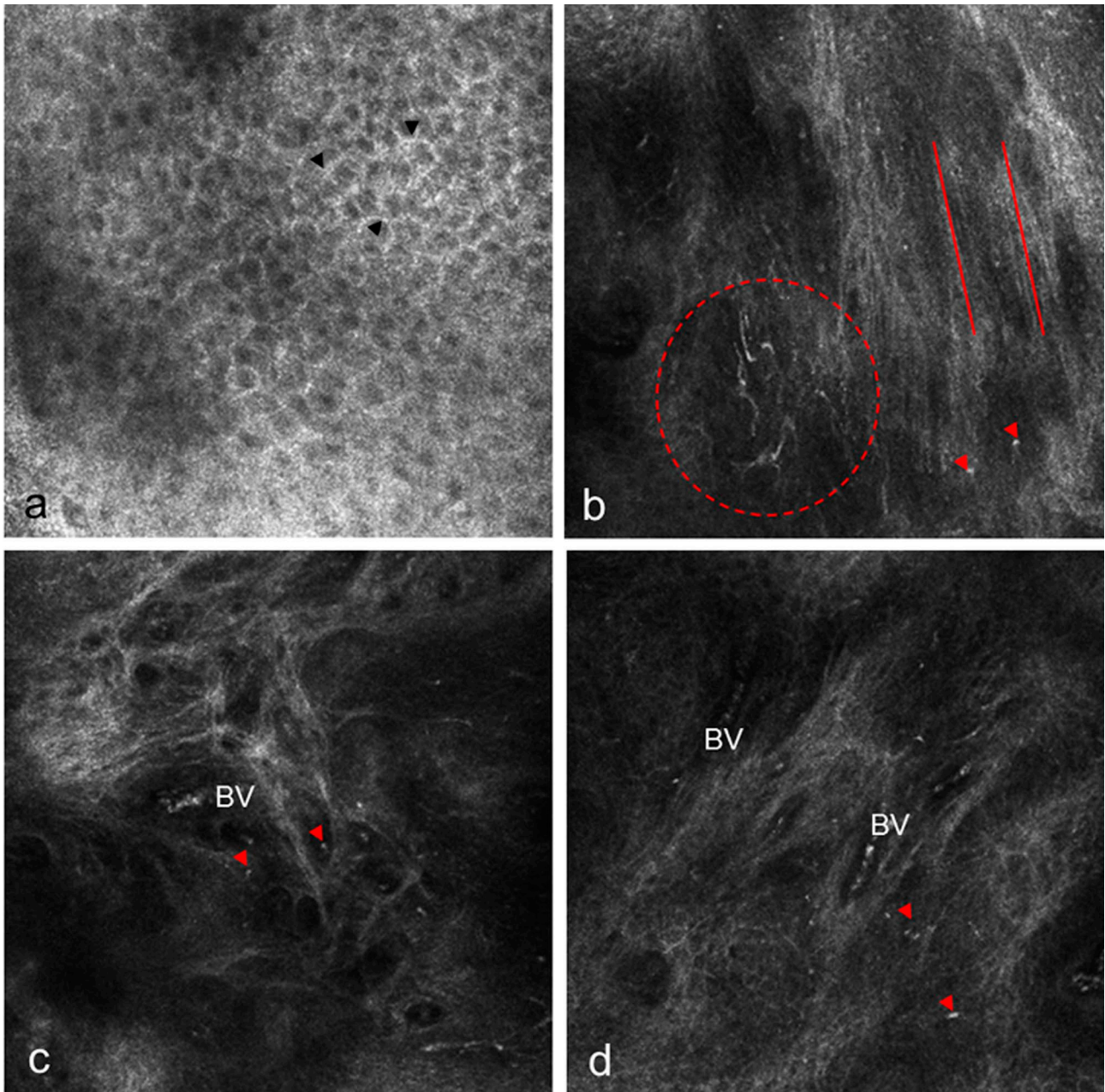
Figure 3. Obtained during the course of treatment of the BCC (shown in Figure 2) with 5% imiquimod cream. Figure 3(a) shows the presence of spongiosis at the granular/spinous layer, which is characterized by accentuation and increased brightness of intercellular borders. Figure 3(b) illustrates persisting polarization (red lines) and appearance of multiple dendritic cells (dashed red circle), which likely correspond to Langerhans cells that are induced by imiquimod treatment. Furthermore, small bright cells corresponding to inflammatory cells are seen (red arrowheads) Figure 3(c) and (d) show dilated blood vessels (BV) and the presence of small bright inflammatory cells (red arrowheads) in the dermis. [Copyright: ©2012 Ulrich et al.]

10 patients and followed up the lesions' evolution for 12 months, identifying two cases of relapse by RCM. In the BCC study, the authors used a liquid nitrogen cryoprobe for burning the area displaying BCC cells, monitoring the effect of the cryotherapy immediately after treatment (5 hours). RCM revealed that tumor clearance was only proven in those lesions showing damage to the upper dermis after 5 h, thus postulating RCM as a tool to determine, almost immediately, the probability of success of cryotherapy. Another study reports on the use of cryotherapy (three cycles separated by three to four weeks) to treat melanoma [32]. Using RCM, the authors discovered residual melanoma cells at the edge of the area that underwent cryotherapy; the procedure was catalogued as a clinical failure and the patient underwent subsequent radiotherapy.

3.4. Anti-inflammatory treatments (Figures 5 and 6)

RCM has also been used to follow up the response to other types of therapy, e.g., NSAIDs. One example is the response of actinic cheilitis (a form of actinic keratosis that affects the
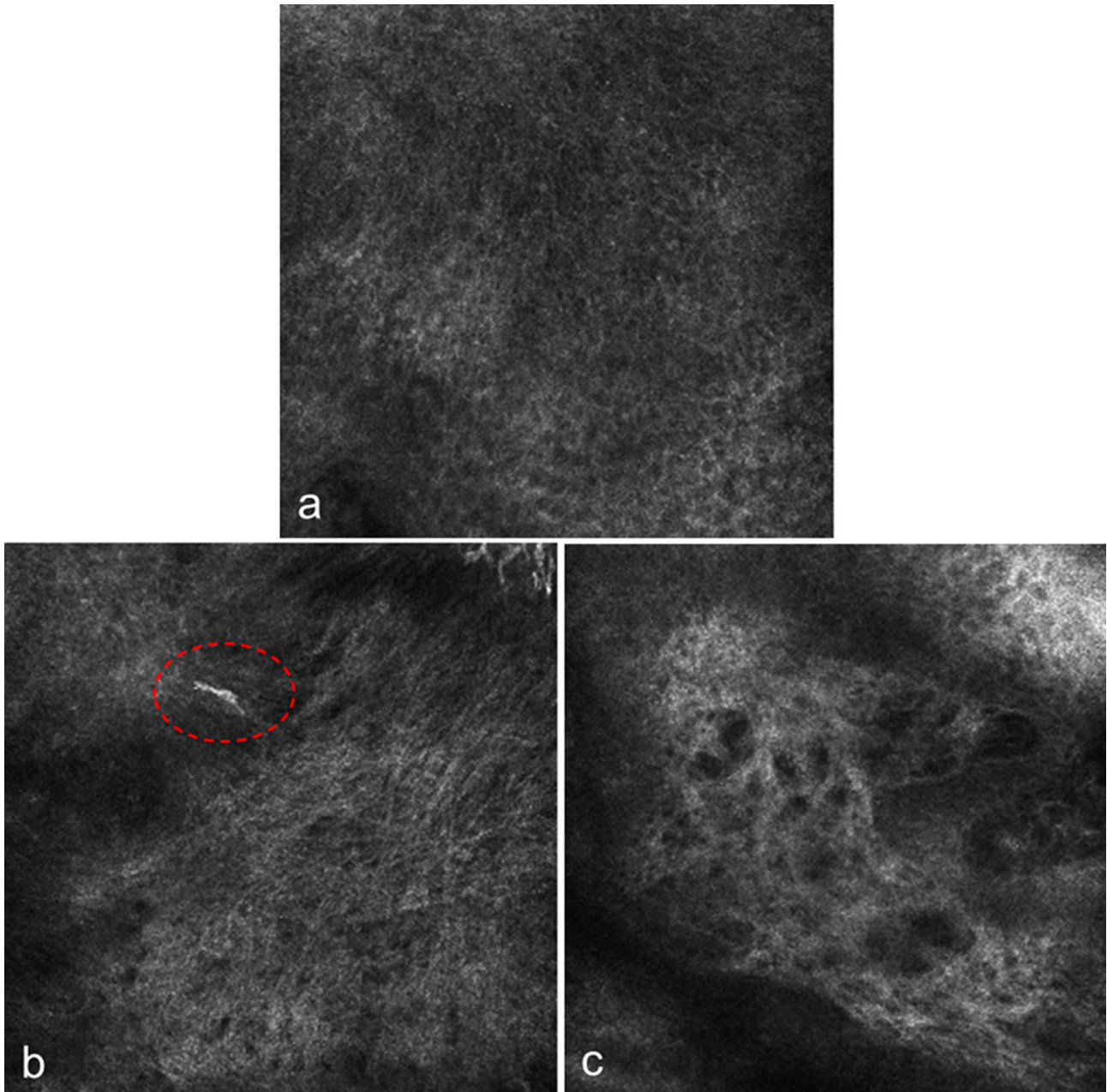
Figure 4. RCM images of the same lesion (shown in Figures 2 & 3) obtained weeks after imiquimod treatment. Figure 4(a) shows honeycomb pattern of the epidermis. Figure 4(b) is showing persistence of dendritic cells in the upper dermis (red dashed circle), that may be observed up to three to six months after treatment with this topical immune-modulator. Figure 3(c) shows normal dermal collagen. [Copyright: ©2012 Ulrich et al.]

lips) to anti-inflammatory (COX-1/COX-2 inhibitor) treatment [38]. The patients were treated with 3% diclofenac in 2.5% hyaluronic acid, twice daily for 90 days. In this study, the authors were able to diagnose actinic cheilitis with RCM in 6/7 (86%) patients. In addition, RCM offered good sensitivity in monitoring the response to diclofenac, revealing regression of the epidermal dysplasia upon consecutive RCM observations.

3.5. Laser-based ablation of non-malignant growths

Laser treatment is a viable treatment option for several benign tumors, e.g., angiomas or sebaceous hyperplasias. RCM has been used to monitor the effect and evolution of laser treatment in these two types of lesions. One clinical case

of cherry angioma was treated with a 585 nm flash lamp-pumped pulsed-dye laser (585 nm, with one pulse of 5 J/cm² using a 5 mm diameter spot) or a 568 nm continuous-wave krypton laser (power=0.75W, 1 mm diameter spot, exposure time = 1 second); the clinical response (from 1 hour up to 4 weeks) was evaluated using RCM [10]. RCM revealed early inflammation followed by resolution of the inflammation and disappearance of the lesion. In another study, the effect of PDL on sebaceous hyperplasia was monitored using RCM. The patients received three stacked, 5 mm wide pulses of a 585 nm pulsed-dye laser equivalent to 7 or 7.5 J/cm² [9]. RCM revealed that, whereas most lesions undergo significant involution over time, recurrence is frequent.
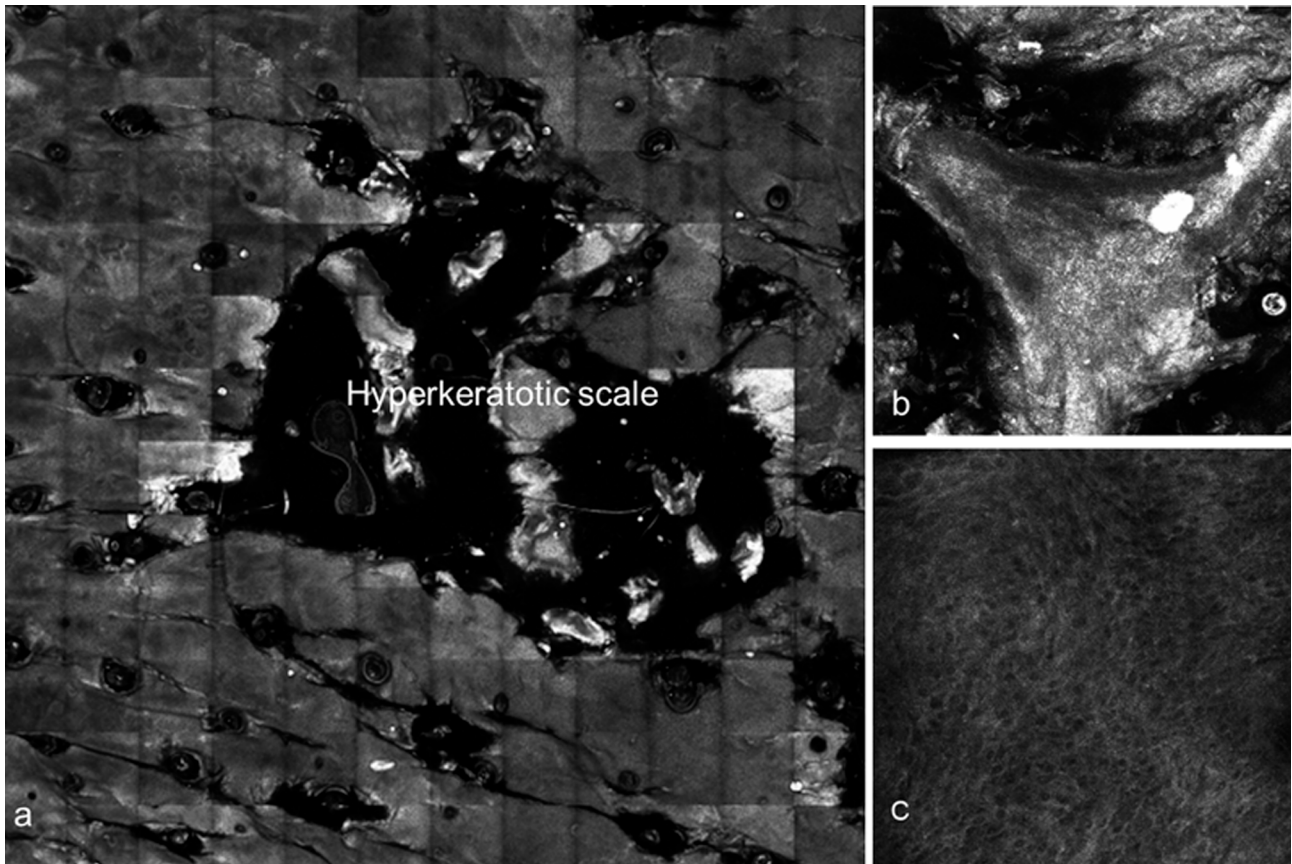
Figure 5. Morphological RCM changes of actinic keratoses during topical treatment with 3% diclofenac in 2.5% hyaluronic acid (twice daily for 90 days). Obtained before initiation of treatment. Figure 5(a) shows RCM mosaic (6 x 6 mm) illustrating typical appearance of actinic keratoses with the presence of superficial disruption and central hyperkeratotic scale. Figure 5(b) shows superficial disruption at the stratum corneum with single detached keratinocytes and hyperkeratosis. Figure 5(c) illustrates atypical honeycomb pattern of the epidermis with atypical keratinocytes as seen in actinic keratoses. [Copyright: ©2012 Ulrich et al.]

3.6. Determination of surgical success and presence of remaining malignant cells

A very important application of RCM as a monitoring tool is the identification of residual neoplastic cells that were left behind at the margins of surgery. These residual cells are the most frequent cause of BCC relapse. A few years ago, a study demonstrated that RCM could be used to identify residual cancer cells after Mohs micrographic surgery of BCC [39]. Although the study included a small group of patients, a strong correlation was observed between the RCM findings and those of conventional histology; thus, the study strongly supports the utility of RCM in scanning surgical margins for residual cancer cells.

4. Conclusion and future perspectives

Although its main application remains diagnostic, the potential of RCM to follow the response to therapy in a completely non-invasive manner is untapped. The major advantages are: RCM is not invasive, allowing repetitive optical tissue sampling; RCM is also independent of contrast agents, yet still provides good resolution, approaching that of histology;

the usefulness of RCM has been improved by the existence of image atlases with histological correlation; and finally, to the trained practitioner, RCM offers ease of use. We identify three major problems, which are being overcome by a combination of ingenuity, hard work and interdisciplinary collaboration. One, technical, is the lack of penetration of RCM light into the deeper layers of the skin; however, technical and optical advances may increase our working depth in the near future. A related problem is resolution. However, the future expansion in clinical use of the new super-resolution confocal microscopes (which rely on statistics to obtain resolutions under 50 nm using light microscopy [40]), promises to be of help. Finally, the data reported so far has been generated based on small-sized patient groups. This is related to the fact that the technique is relatively uncommon in dermatology practice. A combination of good formative opportunities for skin professionals and subsequent word-of-mouth regarding the wealth of high-resolution information this technique offers will increase manifold the patient samples in the near future.

In 2008, a group of basic researchers, clinicians and experts from related fields established an international RCM group (*www.confocal-icwg.com/*). Together, we have under-
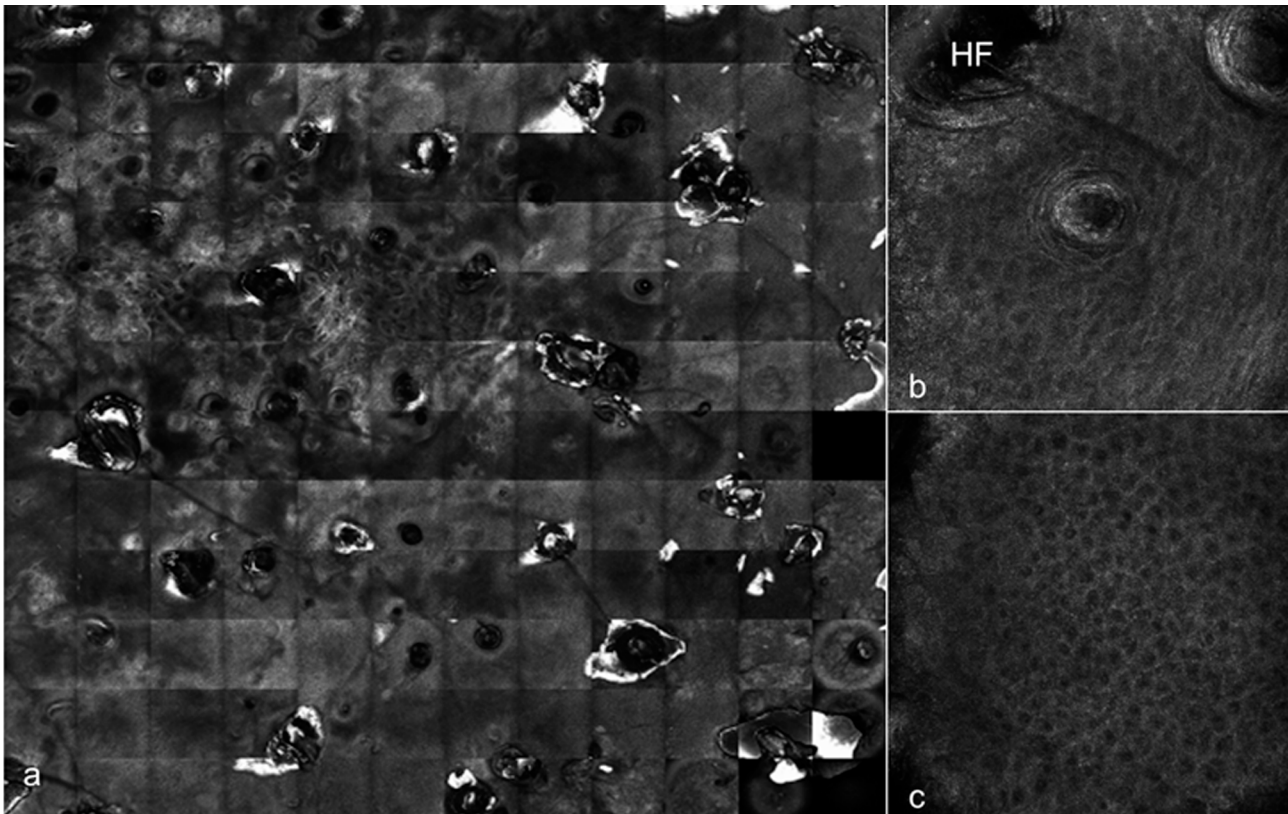
Figure 6. Obtained four weeks after end of treatment with 3% diclofenac in 2.5% hyaluronic acid. Figure 6(a) shows RCM mosaic (6 x 6 mm) with tangential view at the spinous layer as well as the dermoepidermal junction. Disappearance of the hyperkeratotic scale and superficial disruption are noted. Figure 6 (b) shows regular stratum corneum with hair follicles (HF). Figure 6 (c) illustrates regular honeycomb pattern of the epidermis. [Copyright: ©2012 Ulrich et al.]

taken the mission to spread the use of RCM in dermatology. The RCM group provides a forum for free communication of results and for establishment of meaningful collaborations, as well as educates potential new users about the power of this technology. These steps are essential to extend the use of RCM from the academic environment to the general dermatology clinic.

Acknowledgements

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