Current and future applications of confocal laser scanning microscopy imaging in skin oncology (Review)

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Abstract. Confocal laser scanning microscopy (CLSM) is a modern imaging technique that enables the *in vivo* or *ex vivo* characterization of skin lesions located in the epidermis and superficial dermis with a high quasi-microscopic resolution. Currently, it is considered to be the most promising imaging tool for the evaluation of superficial skin tumors. The *in vivo* mode adds the advantage of noninvasive, dynamic, in real-time assessment of the tumor associated vasculature and inflammation. It offers the possibility to repeatedly examine the same skin area without causing any damage and to monitor disease progression and treatment outcome. Furthermore, this novel technology allows the evaluation of the entire lesion and can be used to guide biopsies and to define tumor margins before surgical excision or other invasive therapies. CLSM diagnostic features may differentiate between the various histologic subtypes of skin tumors and therefore helps in choosing the best therapeutic approach. In this study, we present the CLSM characteristic features of the most common melanocytic and non-melanocytic skin tumors, as well as future possible CLSM applications in the study of experimental skin tumorigenesis on animal models.

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1. Introduction

Within recent years, dermatologic imaging technology focused on the development of optical, noninvasive tools to improve diagnostic accuracy and to overcome the disadvantages of histopathological examination. Of all these promising *in vivo* tools, only confocal laser scanning microscopy (CLSM) allows the visualization of cutaneous structures with a resolution that is very close to that of light microscopy, thus performing a skin 'optical biopsy' (1). It enables the noninvasive, virtual sectioning of the skin at different depths, with grey-scale images obtained in horizontal planes (*en face*), parallel to the skin surface and it does not require tissue processing or coloring (2,3). As it allows repeated imaging of the same skin area in real-time, at different time intervals, it is an excellent method for monitoring disease progression and treatment efficacy and studying skin's dynamic behaviour (1,4-10).

Based on the source of image contrast, CLSM can be performed in either fluorescence or reflectance mode. Fluorescence confocal microscopy (FCM) requires the administration of a fluorescent agent to generate contrast (11) and has been used predominantly in experimental studies with promising results in lesional and nonlesional skin (12,13). Reflectance confocal microscopy (RCM) relies on differences

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in the refractive indices of cellular structures (14) and has been extensively applied in the noninvasive assessment of melanocytic (15-18) and non-melanocytic skin tumors (16,19), with features demonstrating a good correlation with dermoscopic and histologic findings. Furthermore, this novel imaging technique proved to be useful for the diagnosis of various inflammatory skin diseases (20), conditions with dermatologic manifestations (21), as well as for the study of dynamic processes like wound healing (22,23), in real-time assessment of blood flow in response to various topical stimuli (10,24) or leucocyte migration (5,6).

Currently, *in vivo* RCM is considered to be the most promising noninvasive imaging technique for the quasi-microscopic morphological and dynamic characterization of superficial skin tumors. Moreover, it helps to define tumor margins before surgical excision or other invasive treatment modalities (19). Moreover, *ex vivo* settings may guide Mohs micrographic surgery (25,26). Recently, novel multilaser devices, combining CLSM in reflectance mode with fluorescence techniques were developed, providing useful additional information when compared with the use of each variant of confocal microscope alone (13).

In this study, we present the RCM characteristic features of the most common melanocytic and non-melanocytic skin tumors (Table I), as well as future possible CLSM applications in the study of experimental skin tumorigenesis on animal models.

2. Application of reflectance confocal microscopy for skin cancer diagnosis

Basal cell carcinoma (BCC) is the most common of all cancers in light-skinned individuals (27) and its incidence is still rising with ~10% each year worldwide (28). Very often, a skin biopsy is needed to confirm the diagnosis, despite its associated invasiveness and costs. Early diagnosis and treatment are of paramount importance because it is locally destructive and can lead to disfigurement (29,30). RCM diagnostic features for various clinical types of BCC have been described (31-36), demonstrating a good correlation with certain dermoscopic and histopathologic findings (37,39). BCC consists of aggregates of basaloid cells at the dermo-epidermal junction or papillary dermis that appear in RCM images either as 'bright tumor islands', cord-like structures surrounded by cleft-like dark spaces, either as 'dark silhouettes', hyporeflective dark areas outlined by bright stromal tissue (36,38-40). These aggregates of basaloid cells often have nuclei that are oriented along the same axis, displaying a 'peripheral palisading' at the periphery of tumor islands (35,36). In the above stratum spinosum, the elongated nuclei of keratinocytes that are polarized along the same axis form the typical 'streaming of the epidermis' (35). Additionally, prominent and tortuous blood vessels with intense leukocyte traffic are present in the dermis and numerous inflammatory cells with various shape and sizes (lymphocytes, melanophages) surround the tumor nests (36). In pigmented BCC, bright dendritic structures that correspond to dendritic melanocytes can be identified inside aggregates of basaloid cells (41) (Fig. 1).

A retrospective, multicenter study evaluated the sensitivity and specificity of five RCM criteria for BCC, including architectural alteration and cellular pleomorphism of the overlying epidermis, areas of refractile tumor cells with elongated, monomorphic nuclei, nuclear polarization, increased dermal vasculature and prominent inflammatory infiltrate (35). Identification of two or more of these five criteria in a sample showed a sensitivity of 100% for BCC diagnosis, whereas four or more of these had a specificity of 95.7% and a sensitivity of 82.9% (35). Of these criteria, elongated, monomorphic nuclei proved to be the most sensitive (100%) and nuclear polarization the most sensitive (91.6%) and specific (97.1%) (35).

Furthermore, this novel technology may be a diagnostic guide in defining the margins of the lesion before surgical excision (42) or laser ablation (29). During Mohs micrographic surgery, FCM proved to be far superior than RCM for tumor margin assessment in BCC (36). Moreover, it offers the advantage of monitoring noninvasive treatment in superficial-type BCC, thus avoiding the discomfort associated with skin biopsy (43,44).

Squamous cell carcinoma (SCC) is the 2nd most frequent non-melanoma skin cancer after BCC and appears dominantly in sun exposed areas. Besides UV exposure, various risk factors, including immunosuppression, viral infections, exposure to chemical agents, neuro-endocrine factors or chronic inflammation, have been proposed to be involved in SCC pathogenesis (45-51). It has various clinical presentations including *in situ* lesions (Bowen's disease), invasive superficial lesions or highly infiltrative lesions (52). Actinic keratosis (AK) is the most common precancerous skin lesion with a risk of progression to a full-thickness SCC estimated at 5-10% (53), but some authors consider it as an early form of SCC as it appears (54). Under RCM evaluation, SCC and hypertrophic AK often present a pronounced hyperkeratosis that limits the depth of penetration considerably (55) and provides whitewashed images because of the strong back-reflectance at the keratin-rich surface of the tumor (39). A more pronounced disarranged honeycomb pattern in the spinous-granular layers and the presence of neoplastic aggregates in the dermis can distinguish SCC from AK (56). Moreover, nuclei are enlarged and pleomorphic (55) and roundish, nucleated bright cells with a pagetoid arrangement are often observed in the suprabasal epidermis. When the thickness of the lesion allows the dermo-epidermal junction imaging, dermal papillae may appear elongated with looping, round vessels inside them (39,57) (Fig. 2). However, in case of infiltrative lesions, the level of invasion is usually inaccessible in CLSM (58). Even with *ex vivo* CLSM during Mohs surgery, the detection of residual SCC is rarely possible also because of the non-reflecting features of keratinization (58,59).

When it comes to RCM evaluation of SCC localized on the lips, distinctive features were described (60). Moreover, RCM evaluation has the potential to distinguish between features of normal mucosa, dysplasia and lip SCC in real-time and therefore may be useful for the preoperative assessment of tumor resection margins (61).

Cutaneous melanoma is one of the most aggressive human malignancies, associated with high mortality rates, despite latest advances in therapy (62-65). An important genetic background and several environmental factors are key players in melanoma development and progression (66-70). Two crucial points have to be taken into account in this form of aggressive

Table I. Summary of the diagnostic reflectance confocal microscopy features for common skin cancers.

Type of skin cancer	Reflectance confocal microscopy features	Author, year (Refs.)
Basal cell carcinoma	Bright tumor islands/dark silhouettes Peritumoral clefting Streaming of the epidermis Prominent and tortuous blood vessels Inflammatory cells ± bright dendritic structures Spoke wheel-like structures	Caruntu *et al*, 2014 (33) Ghita *et al*, 2016 (31) González and Tannous, 2002 (36) Longo *et al*, 2014 (32) Nori *et al*, 2004 (35) Peppelman *et al*, 2013 (34) Segura *et al*, 2007 (41) Stephens *et al*, 2013 (37) Ulrich *et al*, 2010 (38)
Squamous cell carcinoma	Hyperkeratois Disarranged honeycomb pattern with enlarged, pleomorphic nuclei in the spinous-granular layers Round, nucleated bright cells in the suprabasal epidermis Elongated dermal papillae with looping, round vessels	Aghassi *et al*, 2000 (55) Branzan *et al*, 2006 (58) Peppelman *et al*, 2014 (56) Que *et al*, 2015 (39) Rishpon *et al*, 2009 (57)
Melanoma	Pagetoid spread of roundish or dendritic cells in the epidermis Pleomorphic cells and atypical nests at the dermo-epidermal junction Non-edged papillae and atypical nucleated cells in the papillary dermis Poorly defined or absent keratinocytes cell borders	Carrera *et al*, 2012 (80) Guida *et al*, 2015 (16) Pellacani *et al*, 2007 (15) Pellacani *et al*, 2014 (17) Ulrich and Lange-Asschenfeldt, 2013 (79)
Mycosis fungoides	Weakly refractile cells (lymphocytes), Vesicle-like spaces (Pautrier collections) within the epidermis Hypo-refractile papillary rings and dilated capillaries	Agero *et al*, 2007 (90) Fabbrocini *et al*, 2017 (85) Koller *et al*, 2009 (89) Lange-Asschenfeldt *et al*, 2012 (88) Li *et al*, 2013 (87) Mancebo *et al*, 2016 (86)
Primary cutaneous folliculocentric lymphoma	Round-shaped, highly-refractive tumor masses Bright cells of various sizes and numerous bright small cells (lymphocytes) at the periphery of tumor masses	Unpublished study

skin cancer. One is particularly in high-risk patients, where melanomas may be complicated to distinguish from nevi (71) and the fact that numerous biopsy specimens for screening are associated with patient morbidity. Therefore, if a dermatologist is confronted with a lesion obeying the ABCDE rule of melanoma (72) or if the atypical lesion is solitary/is the 'ugly duckling' (73) there are no particular issues for a dermatologist. Conversely, in patients with numerous and clinically atypical nevi, visually identifying the lesion with the greatest atypical features that may represent a new or developing melanoma is almost impossible. Removing high numbers of nevi in such patients for finding one melanoma can be a screening method, but although there are extended publications on how many nevi should be removed in high-risk patients to identify one melanoma (74-77), there are still issues such as removing too few nevi can be associated with overlooked melanomas and/or significant medical system costs (78).

In particular, for this aggressive type of skin cancer RCM allows a noninvasive *in vivo* imaging at cellular-level from superficial melanomas to dermis invading melanomas. This important new tool has an emerging diagnostic role in the characterization of melanomas as a noninvasive *in vivo* histo-morphological analysis and as an added device in following the clinical management of skin cancer patients (79).

In the case of melanoma, the melanocytic lesions have in the upper parts of the tumor pagetoid roundish or dendritic cells in the superficial epidermis, atypical nests at the dermo-epidermal junction, non-edged papillae and atypical nucleated cells in the papillary dermis. The benefit of RCM *in vivo* examination in real-time is important also in particular cases of melanoma like lentigo maligna melanoma and amelanotic melanoma. Moreover, this technology can add information on management of subclinical margins, recurrences, or monitoring noninvasive treatment of tumors (80).

Studies that focused on the application of this technology in melanoma diagnosis have shown that melanocyte-derived tumor cells can be demarcated from non-melanocytic ones. Thus, our experience has shown that, while benign nevi
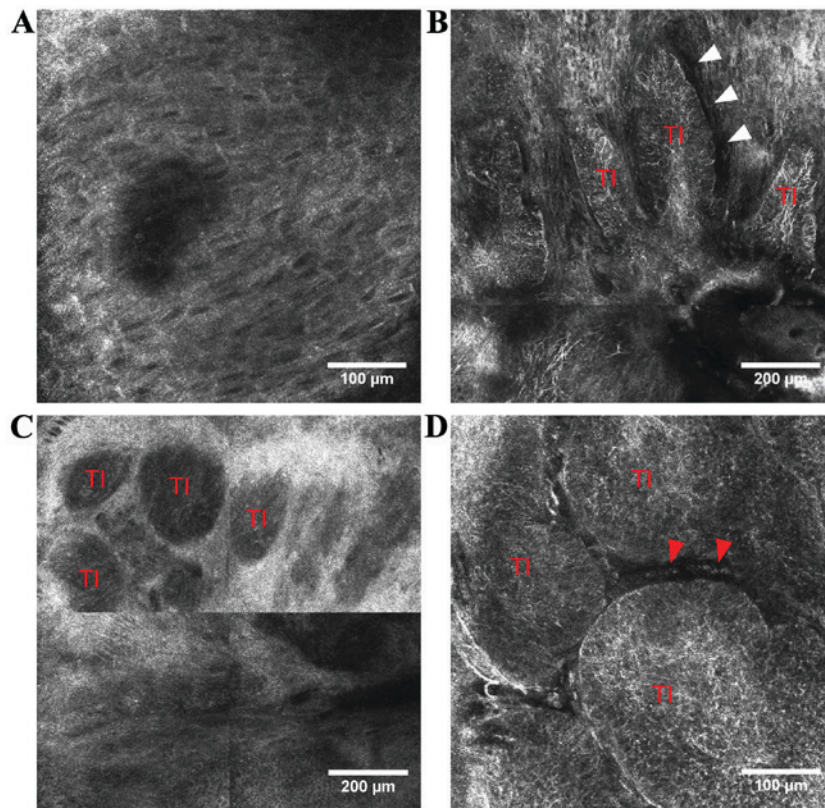
Figure 1. RCM features of BCC. (A) RCM image (500x500 µm) showing polarization of keratinocytes along the same axis forming epidermal 'streaming'; (B) RCM mosaic (1x1 mm) in the tumoral area of a pigmented BCC displaying elongated TI infiltrated by bright dendritic cells, peripheral palisading and peritumoral dark spaces (white arrowheads), also known as 'clefting'; (C) RCM mosaic (1x1 mm) showing 'dark silhouettes' representing TI, in the tumor region of a nodular BCC; (D) RCM image (500x500 µm) of BCC showing TI infiltrated by dendritic cells and blood vessels (red arrowheads) surrounding the neoplastic dermal aggregates. TIs, tumor islands.
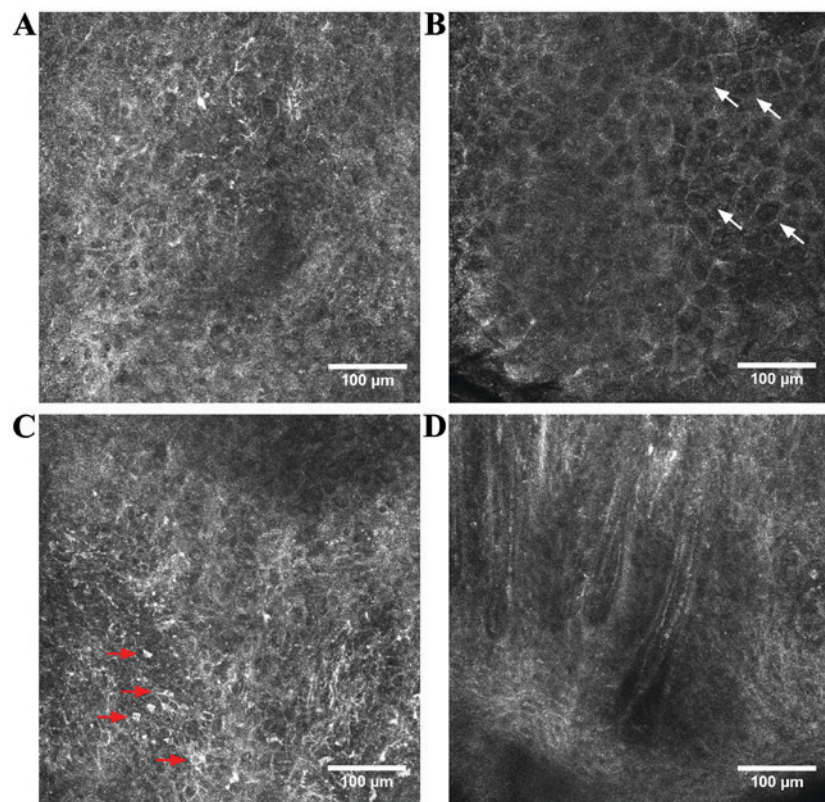

Figure 2. RCM features of SCC. (A) RCM image (500x500 µm) of an atypical honeycomb pattern in the stratum spinosum; (B) RCM image (500x500 µm) of dyskeratotic cells, also known as 'targetoid cells' (white arrows); (C) RCM image (500x500 µm) at epidermal level showing roundish, nucleated, bright cells with a pagetoid arrangement (red arrows); (D) RCM image (500x500 µm) showing looped vessels in the tumoral region of an SCC.
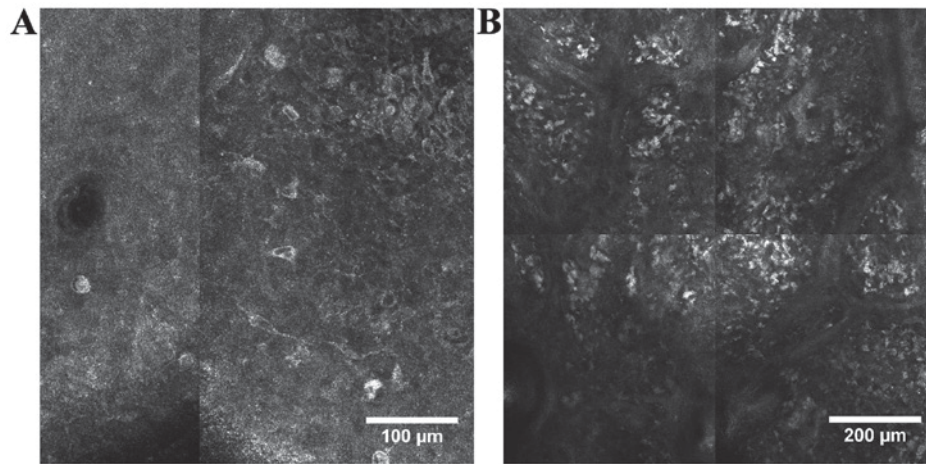
Figure 3. RCM features of cutaneous melanoma. (A) RCM image (500x500 μm) displaying big, roundish and dendritic, bright, pagetoid cells infiltrating the epidermis; (B) RCM mosaic (1x1 mm) showing large heterogeneous nests of melanocytes with the presence of numerous atypical cells in the tumoral region of a cutaneous melanoma.

have monomorphic cells, round to oval in shape, with bright appearance, in melanomas cells are bright, polymorphic and irregular, roundish or with branching dendrites (Fig. 3). In benign nevi, junctional and dermal nevus cell nests can be found, while in melanomas there is a disarray of the melanocytic cell architecture. Keratinocyte cell borders can be detected readily but are poorly defined or absent in melanomas. The horizontal optical sections in RCM offer a better visualization of malignant melanocyte morphology than classical hematoxylin and eosin stained histologic sections (15,81). In addition, based on their cell morphology in RCM, four types of melanomas have been identified, namely dendritic cell melanomas, melanomas with roundish melanocytes, melanomas with predominant dermal nesting proliferation and combined type melanomas, each with different tumor and patient characteristics (15).

More elaborated studies seeking to evaluate specificity and sensitivity of this technology have reported that there is a good differentiation between benign versus malignant tumors. Thus, depending on the observers, the sensitivity ranged from 90.42 to 97.62% and the specificity from 96.67 to 100%. These values generated good performance of the investigation: sensitivity, 94.65%; specificity, 96.67%; positive predictive value 97.50%; and negative predictive value 92.99% (82).

Gathering important information from large studies, this quasi-histological *in vivo* evaluation has no restrictions for age, sex, ethnicity and has a good association with clinical, dermoscopic and histopathologic findings. Therefore, diagnostic accuracy, sensitivity and specificity of the technique were a good backbone to implement it in the diagnosis of melanoma (16). This new technology brings, besides non-invasiveness characteristics, new mapping possibilities of difficult melanomas like lentigo maligna of the face (78).

Cutaneous lymphomas are a heterogeneous group of lymphoproliferative disorders involving the skin that are characterized by clonal proliferation of mature T-lymphocytes (>60% of all cases), B-lymphocytes or NK cells (83,84). Histopathological examination combined with immunohistochemistry of the skin biopsy specimen is the mainstay of the diagnosis, although sequential biopsies are often needed, especially in case of early stage lesions.

RCM has already been reported to be useful for the *in vivo* diagnosis (85-91) and therapeutic follow-up of cutaneous T-cell lymphomas (92), with the majority of studies referring to its most common type, mycosis fungoides (85-90) and one to lymphomatoid papulosis (91).

Mycosis fungoides, early patch lesions in particular, can imitate a wide variety of erythematosquamous skin diseases and its clinical and histopathological diagnosis is often a challenge. Most commonly reported RCM features of mycosis fungoides correlate with histopathologic findings and include weakly refractile cells (lymphocytes) and vesicle-like spaces (Pautrier collections) within the epidermis, hypo-refractile papillary rings and dilated capillaries with thick walls at the dermo-epidermal junction (90). Detection of Pautrier collections with RCM is associated with improved histopathologic diagnosis and presence of TCR gene clonality (86).

The rest of the RCM findings are non-specific and reflect the heterogeneous clinical and histopathologic presentation of the lesions (86). However, *in vivo* RCM seems to be reliable in guiding skin biopsy collection, therefore reducing the number of unsuccessful histopathological examinations for mycosis fungoides lesions (85,87).

In contrast to cutaneous T-cell lymphomas, to date no RCM features have been described for the diagnosis of B-cell lymphomas. Our research team recently described the *in vivo* RCM features observed in primary cutaneous folliculocentric lymphoma lesions (unpublished results). These correlate with histopathology and include round-shaped, highly-refractive tumor masses in the dermis, bright cells of various sizes dispersed throughout the dermis and aggregates of bright small cells (lymphocytes) at the periphery of tumor masses (Fig. 4).

3. Application of confocal scanning laser microscopy for skin oncology research

Skin carcinogenesis is a complex, multifactorial process and the topic of intensive research given the continuously
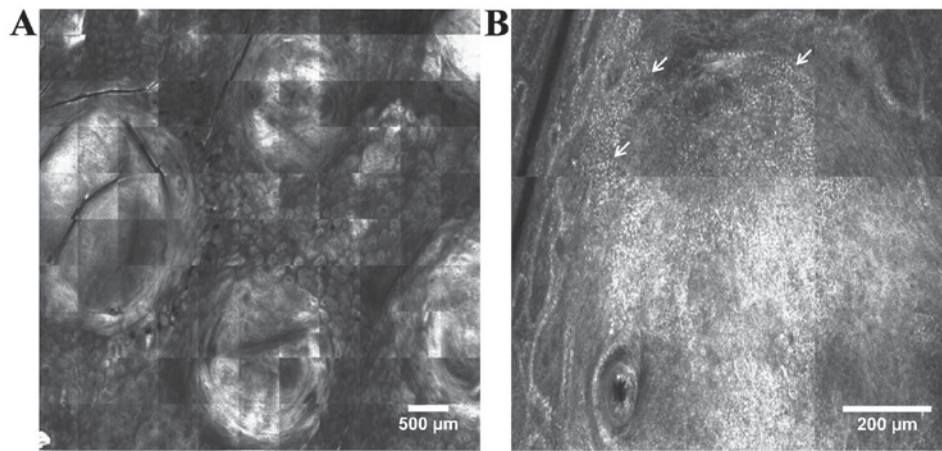
Figure 4. RCM features of cutaneous lymphoma. (A) RCM mosaic (5.5x5.5 mm) showing well-demarcated, round-shaped, highly-refractive tumor masses; (B) RCM mosaic (1x1 mm) displaying aggregates of bright small cells (white arrows) at the periphery of a tumor mass.
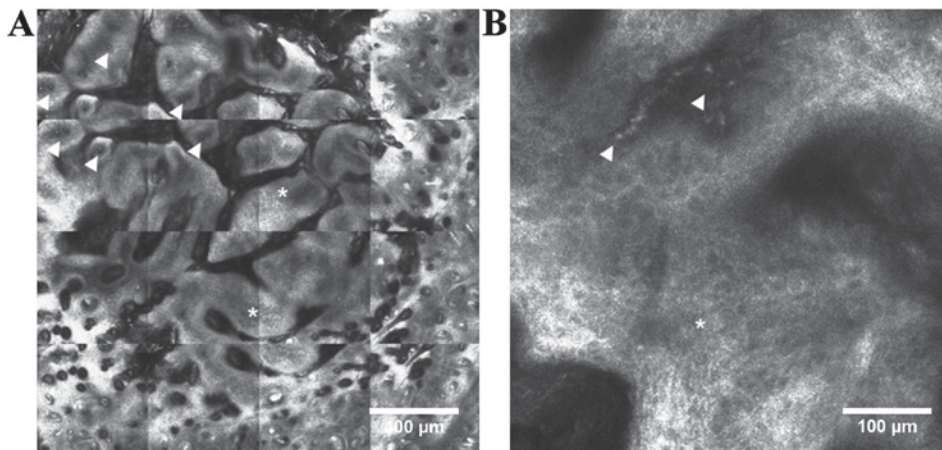

Figure 5. Chemically induced carcinogenesis in CD1-Foxn1nu mouse. (A) RCM mosaic (2x2 mm) showing a tumor with multi-lobular structure with altered keratinocyte architecture (white asterisks) and numerous blood vessels (white arrowheads); (B) detailed RCM image (500x500 μm) showing atypical cells (white asterisk) and an enlarged, tortuous blood vessel (white arrowheads).

increasing incidence of skin cancer. In addition to the recognized genetic and environmental factors (93), prolonged exposure to pro-inflammatory cytokines and chemokines within chronic inflammation is experimentally sustained to favor initiation and progression of skin cancer (94).

Mouse models of chemically induced skin carcinogenesis are one of the most available and cost-effective models to analyze early alterations and pathways involved in skin tumorigenesis (95). The two-stage skin carcinogenesis model has been used to study mechanisms of epithelial cancers (96) and it refers to the two-step topical administration of chemicals to mouse skin for the initiation and promotion phases of skin tumorigenesis. This delimitation of phases allows the observation of premalignant lesions (96) and it offers more reliable results when testing the effects of environmental factors and drugs on skin tumors (95).

In vivo CLSM is a new imaging technology, not yet fully explored for investigating murine skin structures within experimental tumorigenesis. Reflectance mode CLSM was reported to allow real-time observation of abnormal tissue architecture, atypical structures, as well as the blood flow and vasculature that accompanies skin tumors (95) (Fig. 5). Dendritic immune cells are difficult to differentiate from melanocytes under RCM as they have similar morphologic features (97), but activated Langerhans cells seem to have a more superficial epidermal localization (41).

Fluorescence mode CLSM studies have been done on transgenic mice using green fluorescent protein marker to visualize cellular details of the skin (11). As it allows sequential noninvasive examination of the same skin area, CLSM technology seems to be ideal for monitoring tumor progression (98) and therapeutic effects of anticancer agents in mouse models of skin carcinogenesis (95). Recently, a dual mode *in vivo* reflectance and fluorescence CLSM has been developed and holds significant promise for imaging tumor progression in murine skin (98). This system combines the fluorescence contrast of targeted tumor cells with the acquired reflectance contrast of examined cells and tissues and place them within a histologically meaningful framework (98). In addition to the *in situ* visualization of tumor cell proliferation and vascular structures, it has been shown that combined reflectance/fluorescence *in vivo* CLSM has the ability to image dendritic immune cell trafficking to inoculated tumors and to monitor tumor induced immune response in the skin (98).

4. Limitations and future perspectives

Despite the great advantages CLSM adds to dermatological practice, it also has some limitations, the most recognized being the restricted depth of penetration to 200-300 μm, that allows imaging only of the epidermis and upper dermis (3,4). Therefore, the deeper part of the dermis and the hypodermis cannot be visualized using the currently commercially available confocal microscopes. Examination of deeper skin structures could be achieved using higher laser power, but at the expense of damaging the skin area under evaluation (52). There are attempts to develop new devices that improve light collection from the examined plane in order to increase depth of penetration (99). Moreover, examination of skin lesions by means of CLSM is more time consuming than clinical evaluation or dermoscopy and it needs training and experience for the interpretation of CLSM images. Recent technological breakthroughs have led to the development of new, smaller and more practical hand-held devices that offer faster image acquisition and allow the examination of lesions located in less accessible body areas (52). Unlike vertical sections obtained in conventional histology, CLSM enables virtual sectioning of the skin at different depths, in horizontal planes (*en face*), parallel to the skin surface (3). For a better correlation with histology sections, current efforts are aimed at developing devices that could also perform optical sections of vertical planes and then compile 3-D reconstructions of the lesions (100). In addition, CLSM does not require tissue processing or coloring and images are obtained in greyscale, similar to X-rays or ultrasonography (2,3). To improve contrast of epidermal and dermal structures and toward color-enhanced *in vivo* CSLM imaging, fluorescent dyes like indocyanine-green are being tested (101).

5. Conclusions

CLSM is a modern imaging technique that enables the noninvasive characterization of skin lesions located in the epidermis and superficial dermis with a high resolution. Currently, it is considered to be the most promising imaging technique for the quasi-microscopic morphological and dynamic characterization of superficial skin tumors. The *in vivo* mode adds the advantage of noninvasive, dynamic, in real-time assessment of the tumor associated vasculature and inflammation. It allows sequential noninvasive examination of the same skin area without causing any damage and to monitor disease progression and treatment outcome. Furthermore, CLSM technology seems to be ideal for monitoring tumor progression and therapeutic effects of anticancer agents in mouse models of experimental skin carcinogenesis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MAI, CC, ML, DL, MT, SGR, AB, CC, MN, SAZ and DB were responsible for acquisition of references, analysis and systematization of data, and contributed to writing the manuscript and revising it critically for important intellectual content. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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