

Confocal Reflectance Microscopy in Dermatology: Promise and Reality of Non-Invasive Diagnosis and Monitoring

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Summary. *In vivo* reflectance confocal microscopy (RCM) constitutes a novel non-invasive imaging method that enables visualization of cells and structures in living skin in real time with resolution close to histological analysis. RCM has been successfully implemented in the assessment of benign and malignant lesions. It also enables monitoring dynamic changes in the skin over time and in response to different stimuli, such as ultraviolet exposure or specific treatments. The non-invasive nature of this technique allows repetitive sampling without biopsy collection, causing no further damage to the areas under investigation. In this article, I provide an overview of RCM, including the latest technological advances as well as novel applications in the characterization of both normal and pathological skin.

Key words: reflectance confocal microscopy, non-invasive imaging techniques, *in vivo* microscopy, skin cancer.

MICROSCOPIA CONFOCAL DE REFLECTANCIA EN DERMATOLOGÍA: PROMESA Y REALIDAD DE DIAGNÓSTICO Y SEGUIMIENTO NO INVASIVOS

Resumen. La microscopía confocal de reflectancia (MCR) *in vivo* constituye un novedoso método de imagen no invasivo que permite la visualización de células y estructuras de la piel viva en tiempo real, con una resolución próxima al estudio histológico. La MCR también se ha implementado con éxito para la valoración de lesiones benignas y malignas. Asimismo, permite detectar los cambios dinámicos en la piel con el paso del tiempo y en respuesta a diferentes estímulos, como la exposición a la radiación ultravioleta o a tratamientos específicos. La naturaleza no invasiva de esta técnica permite repetir las muestras sin realizar una biopsia, evitando así causar más daño a las zonas que están siendo estudiadas. En este artículo se aporta una visión de conjunto sobre la MCR, incluyendo los últimos avances tecnológicos, así como las aplicaciones novedosas para la caracterización de la piel sana y enferma.

Palabras clave: microscopía confocal de reflectancia, técnica no invasiva, microscopía *in vivo*, cáncer cutáneo.

Introduction

Non-invasive techniques are the Holy Grail of diagnostic procedures. When applied to the field of dermatology, biopsy collection requires extra consideration, i.e. the additional damage caused to the affected area, which has both health and aesthetic ramifications.

The last 15 years have witnessed a surge of novel imaging techniques that provide information of the skin and external mucosa; many of these techniques are non-invasive and enable repetitive analysis of the affected area, conferring

the medical practitioner not only immediate diagnosis capability, but also the ability to monitor the effect of time and treatments on the affected area of the skin without causing further damage and enhancing the precision of prognosis of the lesion. Some of these techniques include dermoscopy¹, optical coherence tomography², high-frequency ultrasound³, magnetic resonance imaging (MRI)⁴, fluorescence-mode confocal microscopy⁵, and reflectance-mode confocal microscopy (RCM). The interest in these novel modalities of evaluation of skin disease is illustrated by the fact that the number of citations in Medline for RCM has passed from 10 in 2000 to more than 250 in 2009.

As of present time, histology is still used as the reference for these applications due to its diagnostic and resolution power, thus an immediate challenge is to establish strong standards for these techniques. *In vivo* RCM offers several

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important advantages over conventional histology. Imaging is painless and non-invasive, causing no tissue damage. The skin is not altered by post-collection processing (fixation, sectioning, and mounting) or staining, which may cause disruption of the native structure of the tissue, thus reducing interpretive ambiguities. Real-time data collection is faster than routine histology, and the same location of the skin can be repeatedly imaged over time to evaluate dynamic changes such as tissue growth, wound healing, lesion progression or response to therapy⁶⁻¹⁰. This is one of the main advantages of the technique compared to conventional histology, which can only reveal information about the tissue at the time of collection of the sample. However, the establishment of tight and reproducible correlations between conventional histology and RCM is an absolute requirement to support the implementation of RCM in general dermatology. In this regard, a recent report establishes a general glossary of terms for the RCM evaluation of melanocytic lesions¹¹, which constitutes a terminology consensus that permits direct correlation of RCM findings with those obtained through classic histology.

Reflectance Confocal Microscopy: Principles and Technology

The principle of confocal scanning microscopy was first described by Marvin Minsky in Harvard as early as 1957¹², but *in vivo* application has required extensive development of light sources and computerization technologies. Dating back to the 1980s, tandem scanning confocal microscopy has been successfully utilized to collect images of human and animal tissue *in vivo*¹³⁻¹⁵. A breakthrough came in 1995, when confocal scanning laser microscopy was used to image human skin *in vivo*¹⁶. Since then, many reports have suggested its possible applications in dermatology, particularly in the diagnosis of skin tumours.

Reflectance confocal microscopy is based on the illumination of a small region within translucent tissue, such as skin or mucosa. The reflected light (reflectance) is sent through a pinhole, which prevents out-of-focus light from reaching a detector, so that only light from the in-focus plane (confocal) is detected. In this manner, whole planes of the sample under study are collected by linear scanning of the point source beam, providing thin sections of horizontal tissue *in vivo*, similar to computerized tomography or MRI. Thus, optical sectioning allows intact tissue to be studied, avoiding invasive damage caused by sample collection for histological analysis.

Light reflection is caused by local variations of the refractive index within the tissue; it also occurs when the size of the tissue elements imaged is similar to the illuminating wavelength. For example, melanosomes produce strong reflectance when illuminated with near-infrared wavelengths

because they contain material with a high refractive index relative to the surrounding skin, and have a size similar to the illuminating wavelength¹⁷.

Technically, confocal microscopes used in dermatology are not very different from their counterparts used in basic sciences. Typically, confocal microscopy employs lasers as source of illumination due to their capability to generate monochromatic, coherent beams. Whereas fluorescent confocal microscopes use He/Ne, Kr or Ar lasers to illuminate fluorescent samples in a wavelength range of 400-700 nm, light transmittance in skin increases from 700-1400 nm¹⁸. Due to the fact that resolution is related to the wavelength of the illuminating beam, higher wavelengths, which have increased light transmittance, have poor lateral resolution¹⁷.

Therefore, confocal microscopes use sources of illumination near the infrared wavelength, 800-1064 nm; available devices have a wavelength centered at 830 nm and less than 30 mW to avoid tissue damage; this setup allows examination at a maximum depth of 200-250 μm , sufficient to image the upper reticular dermis¹⁷.

The laser beam is sent through a beam splitter and a scanning/focusing lens attached to a contact device. Image resolution depends on the precise wavelength used, as well as the optical aperture of the lens and the size of the pinhole.

Newer microscopes include a single optical fiber for the illumination and detection optical paths that eliminates out-of-focus information collected by the lens, simplifying the confocal setup and enabling miniaturization into a hand-held device. A typical example of lens used in RCM is a 30x objective lens of numeric aperture (NA) of 0.9, which provides a lateral resolution of approximately 1 μm and an axial resolution (section thickness) of 3-5 μm ¹⁷.

Skin movement is an important limitation when imaging *in vivo*. To reduce motion blur and contain the water or gel interface when imaging, a skin contact device is used. Most typical contact devices consist of a metal ring that is attached to the skin with adhesive and to the lens by means of a magnetic or snap mechanism. In addition to its function to stabilize the setup, it also holds the immersion medium. These media are water or water-based gels, since their refractive index is very similar to that of human epidermis (1.33 to 1.34), which reduces the spherical aberration of the beam passing through air.

Images are obtained after sequential illumination ("scanning") of multiple pixels within the focal plane, generating a 2D mosaic image, which enables evaluation of architectural patterns. Scanning of consecutive planes potentially allows the generation of 3D maps of the skin. Another application is the collection of time-lapse images of the same plane to produce movies that reveal dynamic events such as blood flow^{6,8,10,16}.

Applications of Reflectance Confocal Microscopy

Diagnosis

The immediate application of RCM derives from the visualization of normal and pathological skin, which bears enormous diagnostic promise. An obvious requirement is the establishment of clear correlations between RCM images and histology to disambiguate the information collected and to allow accurate diagnosis.

Normal Skin: Correlation with Histology

The application of real-time RCM to the study of the skin has two main differences compared to classical histology: the image represents horizontal or *en face* sections; in addition, collected images are transformed into intensity maps (brightness grayscale) that represent the differential reflectance of the different structures of the skin (fig. 1).

The most superficial images correspond to the stratum corneum. It consists of large (10–30 μm), anucleated, polygonal corneocytes that are very bright due to the difference between the refractive indices of the immersion medium and the stratum. These cells are grouped in “islands” separated by skin folds, which appear very dark, compared to the cells.

The stratum corneum overlies the next two layers, the stratum granulosum (25–35 μm) and the stratum spinosum (15–25 μm). The stratum granulosum consists of two to four layers of cells; nuclei appear as dark central ovals, whereas the surrounding cytoplasm is bright and grainy (fig. 1A).

The stratum spinosum consists of a tight honeycomb pattern of smaller cells with clearly delineated cell edges.

The deepest layer of the epidermis is the basal layer (7–12 μm). Basal cells are very bright due to the “melanin hats”, which are supranuclear melanin aggregates. In this layer, melanin-rich melanocytes and pigmented keratinocytes appear as isolated, bright ovals¹⁹.

The suprapapillary epidermal plate at the dermoepidermal junction is visualized as rings of bright basal cells surrounding dark dermal papillae (fig. 1B). These areas also show a central area of blood flow with papillary dermal vascular loops¹⁷.

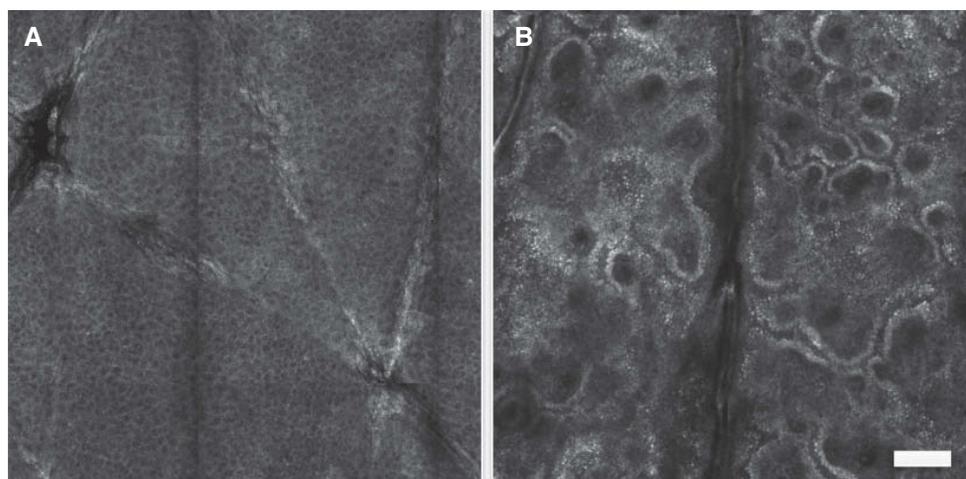
The papillary dermis is visualized as a network of reticulated fibers with central capillary vessels. The reticular dermis appears as a thick reticulated pattern because of the presence of collagen bundles and elastic fibers. Other structures can be visualized such as eccrine ducts, which appear as bright centrally hollow structures that spiral through the epidermis and dermis, and hair shafts with pilo-sebaceous units. These appear as hollow structures with elliptical elongated cells at the circumference and a central refractile long hair shaft.

The appearance of normal skin varies according to the anatomical site, sun-exposure, skin colour, age, and physiological condition being imaged²⁰. Sun-exposed or darkly pigmented skin, for example, is brighter because of increased pigmentation at the basal layer.

Non-Pigmented Lesions

The characterization of skin neoplasms using RCM is an important area of clinical research with potential for non-invasive diagnosis and management of a variety of skin cancers. The advent of newer, less invasive topical therapies such as topical imiquimod or photodynamic therapy demands the development of non-invasive diagnostic tools to identify tumour subtypes and tumour margins accurately, and to monitor the response to treatment.

Figure 1. Normal skin.
A. One millimeter mosaic showing the normal appearance of stratum granulosum and spinosum composed of 15 to 35 μm polygonal cells with dark nuclei and bright and thin cytoplasm giving a honeycomb pattern.
B. Mosaic at the dermo-epidermal junction level showing rimming of dermal papillae by a monolayer of uniform refractile cells. Scale bar, 100 μm .



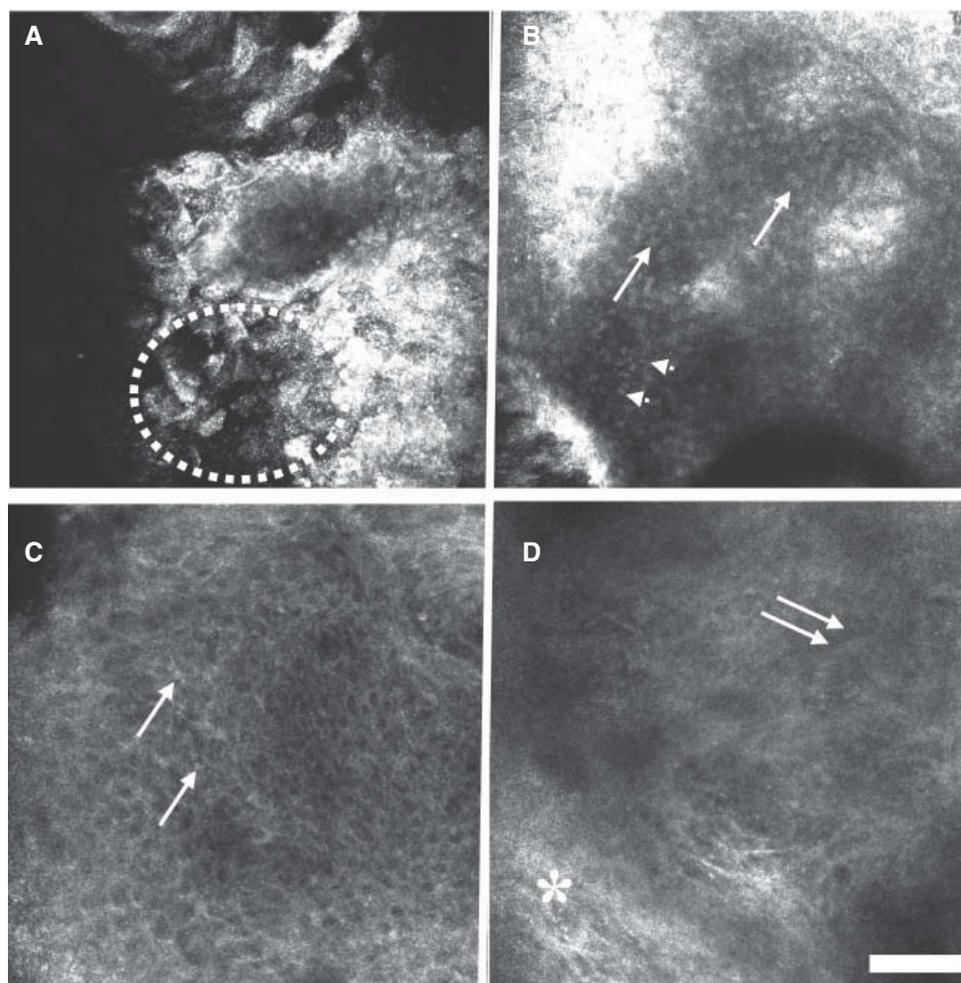


Figure 2. Squamous neoplasia. A. Stratum corneum shows disruptive changes with individual corneocytes (dashed circle). B. Parakeratosis (arrows) and impetiginization of stratum corneum (arrowheads) are also elucidated. C. Malpighian compartment shows keratinocyte disarray, spongiosis and exocytosis (arrows). D. Dermal level images show tortuous small canalicular structures (arrows); scattered round to oval refractile cells—inflammatory cells; and a thickened, moderately refractile lacy background stroma, compatible with solar elastosis (*). Scale bar, 100 μ m.

Epithelial Cancer

Actinic Keratosis

Actinic keratoses (AKs) are the most common precancerous lesions that affect fair pigmented skin overexposed to UV light. AKs are keratinocytic dysplasias (fig. 2); they can evolve into squamous cell carcinomas (SCC). RCM features of AK include irregular hyperkeratosis with disruption (fig. 2A) and parakeratosis (fig. 2B), architectural disorganization and nuclear enlargement and pleomorphism of epidermal cells^{21,22} (fig. 2C). RCM images of AKs show thick bundles consistent with solar elastosis (fig. 2D). The shallow depth of the illuminating wavelength prevents accurate visualization of the dermo-epidermal junction, particularly in hyperkeratotic lesions, which has proven troublesome to distinguish between hypertrophic AK *vs.* SCC. A recent study has revealed the presence of multilocular preneoplastic changes in areas around the site of the lesion, with accuracy around 95%^{23,24}.

Squamous Cell Carcinoma

Squamous cell carcinoma is the second most common skin cancer, and in many cases develops from AK. The confocal features of SCC include architectural disarray and nuclear enlargement with pleomorphism in the stratum granulosum and stratum spinosum, as well as modification of vascular patterns and keratin pearls^{21,25}. Currently, RCM does not enable distinguishing between hyperkeratotic AKs and SCC or between superficially invasive SCC and SCC *in situ* (Bowen's disease), as the dermo-epidermal junction cannot be clearly visualized. RCM has been applied to the diagnosis of oral cavity neoplasms. RCM showed densely packed, pleomorphic tumour nuclei, and epithelial disarray, as well as inflammation. *In vivo*, imaging was possible up to 490 and 250 μ m in the lip and tongue, respectively^{26,27}.

Basal Cell Carcinoma

Basal cell carcinomas (BCC) is the most common skin cancer in humans; RCM features of BCC have been defined: they include islands of monomorphic elongated tu-

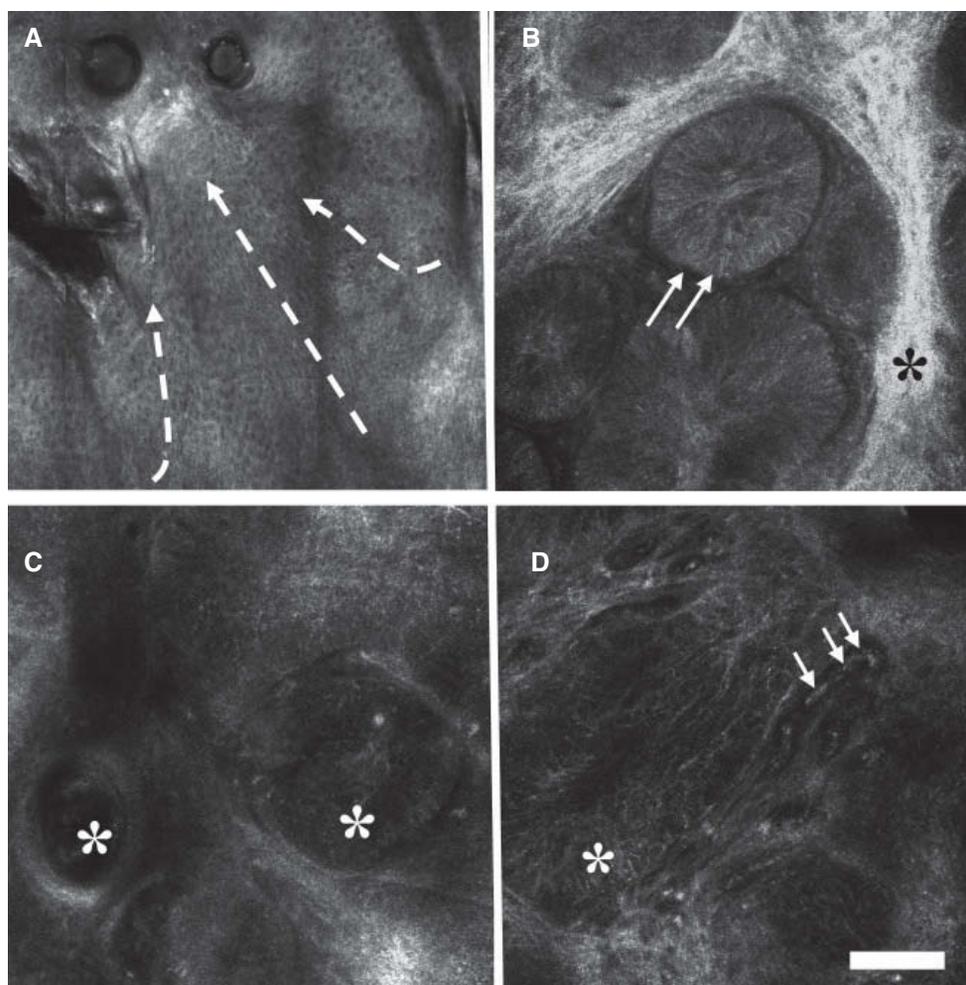


Figure 3. Nodular basal cell carcinoma (BCC). A. Image at the epidermal level showing polarized cell pattern. Population of elongated monomorphic nuclei polarized along the same axis (dashed arrows) manifested as nuclear streaming. B. BCC islands surrounded by bright fibrillar stroma (*). Note palisading of peripheral BCC cells (arrows) and dark cleft-like spaces surrounding the island. C. Hyporefractile aggregates of tightly packed cells forming nodules also termed “dark silhouettes-like islands” (*) and surrounded by bright stroma. D. Dark silhouettes like island (*) in close proximity to a dilated blood vessel (arrows). Scale bar, 100 μ m.

mour cells with their nuclei oriented along the same axis (basaloid nuclei)²⁸ (fig. 3A).

Nodules or islands of tumour cells are seen as distinct aggregates of tightly packed cells, frequently surrounded by cleft-like dark spaces and stromal tissue (fig. 3B) showing a peripheral row of tumor cells containing elongated monomorphic nuclei arranged in parallel to one another and perpendicular to the edge of the tumor aggregate (palisading). In many BCCs, islands may be seen as hyporefractile areas compared to the surrounding stroma (termed dark silhouettes-like islands) (fig. 3C). The area also displays prominent, enlarged blood vessels, in which leukocyte rolling can be occasionally observed; other inflammatory cells can be observed among tumour cells (fig. 3D). Other common features include keratinocyte disarray, epidermis de-structuring and reactive fibrosis; these are usually due to actinic damage and/or reactivity to the tumour.

A recent study has evaluated the sensitivity and specificity of five independent morphological parameters (presence of basaloid nuclei; nuclei polarization; inflammatory infiltrate; increased vasculature; actinic damage) by RCM

for the diagnosis of BCC in 152 lesions²⁹. Of these, polarized nuclei exhibited high sensitivity and specificity; other parameters such as elongated monomorphic nuclei were very sensitive, but specificity was below 75%; correlation of parameters showed that presence of two or more parameters was 100% sensitive, and four or more was 95.7% specific. These results exhibited little variability across BCC locations and subtypes.

RCM may be also useful to non-invasively assess the response to cryotherapy or new therapies such as topical imiquimod or photodynamic therapy^{30,31}.

Mycosis Fungoides

RCM has also been used to visualize epidermotropic lymphocytes, Pautrier’s microabscesses, spongiosis, and tagging of the dermo-epidermal junction by lymphocytes³². Some features include darkening of basal cells around the dermal papillae, corresponding to inflammatory infiltration of the basal layer, and dark spaces filled with dim, small, oval cells, corresponding to Pautrier’s microabscess-

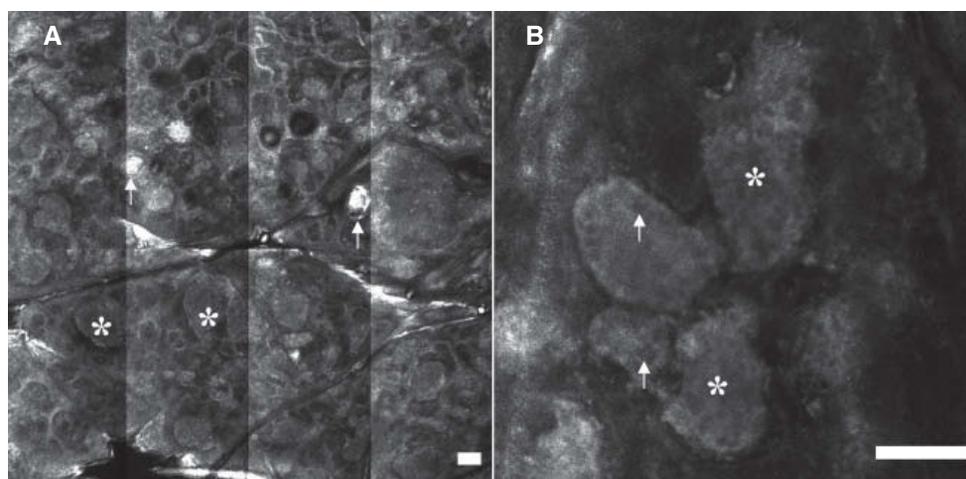


Figure 4. Atypical nevus. A. Two millimeters mosaic at the upper papillary dermis showing multiple dense dermal nests (*) with a moderate atypical architecture and several inclusion cysts (arrows). B. Image denotes dense dermal nest (*) with some atypical nevomelanocytes regarding morphology and brightness (arrows). Scale bar, 100 μm .

es. The latter was typically limited to plaque-type mycosis fungoides (MF). Some of these features are also observed in lichenoid dermatitides and allergic contact dermatitis, making a clear diagnosis difficult.

Pigmented Non-Melanocytic Lesions

Seborrheic Keratosis

Seborrheic keratosis is a common benign epidermal proliferation, sometimes pigmented. RCM reveals marked acanthosis, including epidermal thickening without keratinocytic disarray and long, parallel, dermal papillae; the lesion is usually well delimited by bright rings of basal keratinocytes (*edged papillae*). Pigmented seborrheic keratoses include bright cell clusters (melanophages) and dark canalicular structures, i.e. blood vessels³³.

Dermatofibroma

Dermatofibroma (DF) is a benign dermal proliferation of histiocytes, typically seen in young adult women. Dark pigmented lesions are similar to dysplastic nevi or melanoma. RCM reveals a normal epidermis with homogeneously bright papillary rings and bright keratinocytes above the dermoepidermal junction, corresponding to pigmentation of the basal layer. Moreover, the dermis shows bright collagen bundles corresponding to fibrotic stroma³⁴. Whereas analysis of DF by RCM is hampered by the fact that the spindle fibroblast-like cells, histiocytes and sclerotic stroma are too deep in the reticular dermis, it may still be useful to distinguish it from cutaneous tumours, especially when DF is darkly pigmented and resembles melanocytic lesions.

Pigmented Basal Cell Carcinoma

Pigmented BCC is a variant of BCC that may be difficult to distinguish clinically from benign pigmented lesions or melanoma^{35,36}. RCM features are similar to those previously described for BCC. Melanin distribution is not homogeneous; it is present in benign epidermal keratinocytes, melanocytes interspersed among tumour cells, and in melanophages in the papillary dermis, particularly compared to the surrounding edematous or mucinous dermal stroma of BCCs under RCM.

Pigmented Mammary Paget Disease

Pigmented mammary Paget disease is a rare variant of mammary Paget disease, clinically and histologically similar to melanoma. RCM reveals the presence of large atypical cells resembling pagetoid melanocytosis observed in malignant melanocytic lesions; as well as epidermal disarrangement and dedifferentiation of the papillary edges³⁷. Whereas these data suggested a superficial spreading melanoma, immunohistochemical analysis provided conclusive evidence because most of the Paget cells were positive for *c-erb-b2*.

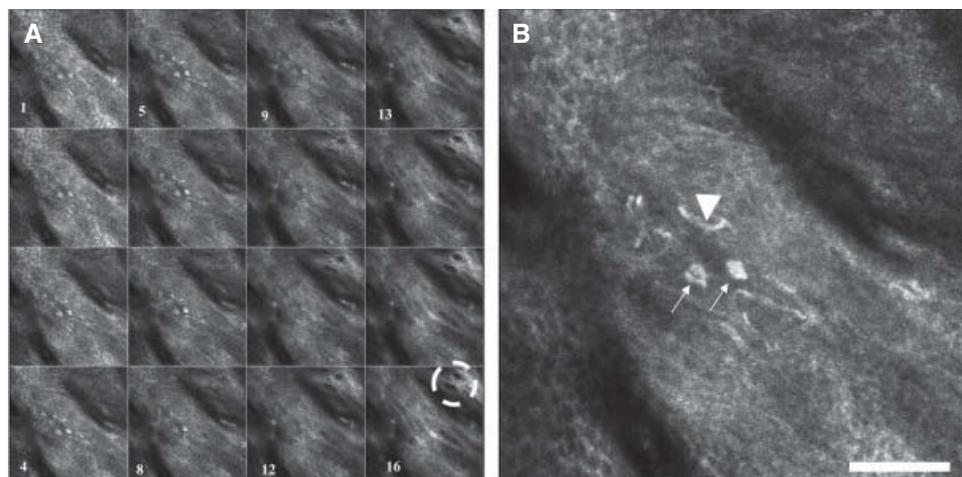
Melanocytic Lesions

RCM is a particularly useful tool to diagnose melanocytic lesions due to the high contrast provided by melanin-containing cells.

Melanocytic Nevi

RCM features of common melanocytic nevi include the presence of a homogeneous population of monomorphic,

Figure 5. Malignant melanoma. A. Stack of consecutive images labeled as 1-16 (2 mm distance) showing the presence of refractile cells and dendritic figures in suprabasal location. Note in images 9-16 dermal papillae without papillary rings (non-edged papillae) (dash circle). B. Focal loss of the honeycomb pattern and presence of highly refractile cells (arrows) and dendrites (arrowhead) in suprabasal location. Scale bar, 100 μm .



oval bright cells with centered nuclei (nevomelanocytes)^{19,38}. Benign nevi exhibit unaltered spinous and granular layers, consisting of homogenous, bright, well-delineated keratinocytes (cobblestone pattern). Dermal papillae are uniformly distributed and delineated by a belt of small, normal melanocytes and basal keratinocytes (*edged papillae*). Junctional nevi are characterized by nevomelanocytes at the dermoepidermal junction, typically surrounding dermal papillae³⁸. In compound nevi, clusters (“nests”) of nevomelanocytes (fig. 4) may be observed at the dermoepidermal junction, often near blood vessels. In both types of nevi, single-cell or clusters of melanocytes can be found in higher layers of the epidermis, but the architecture of the epidermis remains otherwise unchanged.

Dysplastic Nevi

RCM features of dysplastic nevi include irregular shape of dermal papillae and bright rings of keratinocytes. Nevomelanocytes in dysplastic nevi are heterogeneous in brightness, size and shape; they tend to be round or oval resembling those in common nevi, rather than dendritic as in melanomas^{19,38}. Atypical melanocytes appear as isolated large epithelioid cells, with peripheral nuclei, and are weakly bright (fig. 4B)³⁹. Atypical keratinocytes lose their demarcation at the spinous layer and exhibit dendrite-like projections; “melanin dust” (high refractive granular particles that probably represent melanin bodies) is occasionally observed. Facial atypical nevi typically exhibit disruption of the dermoepidermal junction and focal loss of the cell-cell keratinocytic boundaries⁴⁰.

Melanoma

Melanoma is a malignant proliferation of melanocytes. Prognosis is related to the depth of invasion. Superficial

spreading melanomas are characterized by superficial growth followed by vertical invasion and dermal growth. They account for roughly 70% of diagnosed melanomas. Nodular melanoma accounts for 15% of diagnosed melanomas and is the most aggressive variant. Lentigo maligna accounts for 10% of diagnosed melanomas, whereas 5% are acral lentiginous melanomas^{41,42}. The first three classes have been investigated using RCM. Finally, amelanotic (or amelanocytic) melanoma is characterized by cells that do not produce high amounts of melanin.

Superficial Spreading Melanoma

RCM features of superficial spreading melanoma include the presence of enlarged atypical nucleated cells with pleomorphic morphology, variable brightness and angular nuclei, which may be found in several layers of the epidermis (pagetoid dissemination) (fig. 5) and in the dermis. These cells are oval, fusiform or coarsely dendritic, and bear eccentrically placed large dark nuclei. The normal morphology of the stratum spinosum is also altered, including indistinct cell edges and bright, grainy particles (“melanin dust”)³⁹. These alterations in suprabasal layers are defined as keratinocytic disarray, including atypical cobblestone patterns. In basal layers, cells may be grouped, simulating a dysplastic nevus, or isolated. The architecture of dermal papillae is also in disarray, asymmetrical in size and brightness, and includes poorly defined borders (*non-edged papillae*) (fig. 5A) and even sheet-like structures^{43,44}. In the dermis, cerebriform clusters (nodular areas) or sparse cell nests are observed, bearing non-demarcated cell borders and pleomorphism, and surrounded by thick (1-3 μm) “melanin dust” along the epidermis⁴⁵.

On the face, RCM reveals atypical cobblestone patterns with asymmetrical bright boundaries, surrounding follicular apertures with bright dendrites or atypical nucleated bright cells. These patterns are clear indications of malignancy.

nant melanoma. The architecture of dermal papillae is similarly disrupted in lentigo maligna melanoma, including bright anucleated cells (melanophages) within the dermis.

Nodular Melanoma and Nodular Areas of Superficial Spreading Melanoma

Nodular areas of superficial invading melanoma or *bona fide* nodular melanomas have not been studied as thoroughly as superficial melanomas by RCM. The features of *bona fide* nodular melanoma are not as discernible as in superficial melanoma, since epidermal disarray and pagetoid melanocytosis are not apparent; these features are present in superficial invading melanoma. At the dermoepidermal junction, nodular areas of *bona fide* nodular and superficial spreading melanomas have similar RCM features. Papillary apertures are surrounded by bright rings of cells not apparent in non-pathological nodular areas. RCM also reveals pleomorphic cells with bright cytoplasm and dark nuclei (atypical melanocytes) distributed in sheet-like structures between the basal layer and upper dermis, sometimes aggregated in heterogeneous clusters⁴⁵. At the reticular dermis, RCM reveals aggregates of dark, nucleated lobe-shaped cells called cerebriform nests, which correlate with deep melanoma infiltration. Other structures revealed by RCM are plump-shaped dermal melanophages and enlarged blood vessels situated below the nodular areas, consistent with angiogenesis in thick malignant lesions⁴⁵.

Since the presence of nodular features correlates with deep melanoma infiltration, such RCM features require further validation and investigation aimed at developing prognostic uses.

Lentigo Maligna Melanoma

Many of the RCM features of lentigo maligna melanoma are similar to superficial spreading melanoma. They include general or focal disruption of the epidermal layers, with numerous scattered large pleomorphic cells with prominent dendritic processes; atypical bright cells around hair follicles; plump-shaped melanophages and nests of atypical nucleated cells in the papillary dermis.

Amelanotic Melanoma

Features typical of melanoma can also be detected in amelanotic melanoma using RCM⁴⁶, such as the presence of melanocytes, detected by the size and presence of melanin in pre-melanosomes. RCM revealed bright round, oval or fusiform cells, sometimes with dendritic processes. These cells were present either isolated around the dermoepidermal junction and spinous layer, or in confluent,

poorly-defined groups. In addition, the architecture of the epidermis was in disarray and distinct cell borders were lacking. These RCM features correlated well with conventional histology.

RCM-Based Diagnosis of Melanoma

Criteria to accurately distinguish between benign and malignant pigmented lesions need to be further defined, developed, refined and tested; however, it is already possible to distinguish between benign nevi and atypical or malignant melanocytic lesions. A recent study described the application of RCM to distinguish between benign and melanocytic skin tumours. That study found the images derived from the dermoepidermal junction to be the most useful for diagnosis. Melanocytic cytomorphology and architecture, as well as assessment of the edges of keratinocytes were the most useful markers. On the other hand, evaluation of melanocytic cell brightness and dendrite-like structures showed no relevance regarding diagnostic value. It was interesting to note that evaluators without skin histopathological training performed better than trained dermatopathologists, which suggests that the two approaches are not completely equivalent. Also, the use of a diagnostic algorithm for evaluation of RCM images has been recently proposed⁴⁷. This algorithm has two major and four minor RCM criteria associated to malignancy. Major criteria (2 points) include the presence of *non-edged papillae* and cytological atypia, whereas minor criteria (1 point) include the presence of round, bright, and nucleated cells within the epidermis, widespread pagetoid cells, cerebriform clusters and bright nucleated cells within the dermal papilla. Lesions with a score equal to or greater than three showed 97% sensitivity and 72% specificity⁴⁷.

Infiltration of the epidermis by melanocytes (pagetoid melanocytosis) is considered a relevant factor for diagnosis of melanoma, although it is also occasionally present in benign melanocytic lesions⁴⁸. RCM evidences pagetoid melanocytosis in the external layers of the skin, indicating that melanoma diagnosis should be considered, although it cannot be excluded in the absence of pagetoid cells, which are absent in more than 10% of malignant lesions⁴⁷. Similar results are shown for eczematous lesions⁴⁹. Thus, although the presence of pagetoid dissemination is relevant, observation of round pagetoid cells is more specific and it can be considered a further criterion for melanoma.

Guidance for Collection of Biopsy Samples

RCM has shown preliminary promise in the guidance of collection of biopsy samples. A recent report describes the

use of RCM in collecting samples for the diagnosis of *micosis fungoides*, a hard-to-diagnose lesion due to the lack of clear histological markers and clinical diversity, which frequently requires multiple sampling for accurate diagnosis³². RCM can also be useful to collect samples from aesthetically compromising areas, such as the face. For example, RCM analysis of lentigo in the face allows a clear, non-invasive diagnosis of solar lentigo, and reduces the size of the sample required to diagnose lentigo maligna melanoma^{50,51}. Finally, RCM reduces the need of collecting samples in the evaluation of nevi, most of which are benign upon histological examination.

Assesment of the Margins and Adjunct to Surgery

RCM can be used to define the margins of a lesion before surgical or non-surgical therapy. This is particularly helpful in margin assessment of tumours with radial growth phases, including lentigo maligna melanomas⁵² or some basal cell carcinomas⁵³, or hard-to-detect tumours, such as amelanotic melanomas⁴⁶ or infiltrative basal cell carcinomas²⁸. The main caveat is the limited depth that RCM enables, which prevents accurate imaging at depths below the superficial dermis. Other caveats include lack of contrast, which can be potentially resolved by the future development of exogenous contrast agents⁵¹.

Also, RCM can contribute to the rapid establishment of tumour margins by examining excised specimens during procedures such as Mohs micrographic surgery^{53,54}. Mohs micrographic surgery is a histologically guided method of removing skin cancers aimed to preserve the maximum amount of unaffected tissue. Consecutive thin layers of skin are examined by conventional histology to determine if cancerous cells remain. Selective removal of the residual tumour is performed until tumour-free margins are obtained. RCM of *ex vivo* unprocessed tissue can detect neoplastic cells by using 5% acetic acid and cross-polarized illumination. This technique makes the neoplastic nuclei more bright and surrounding dermis dark. Recently, it has been demonstrated that RCM may be useful to examine non-melanoma skin cancers in *ex vivo* tissue during Mohs micrographic surgery without frozen sections^{53,54}. This novel application of RCM offers the advantage of rapid visualization of margins compared to permanent sectioning and confers the capability to do special stains in ambiguous areas⁵⁵. RCM has also been used *in vivo* during Mohs surgery to help visualize the margins of BCC and melanoma tumours⁵⁶. However, poor visualization of non-flat wounds, wound fluid interference, and limited depth are caveats that need to be solved before implementing the routine application of RCM jointly with Mohs surgery.

Evaluation of Response to Treatment

Biopsy is the most common way to assess histological changes during and after treatment. RCM enables repetitive analysis of the same skin lesion or site. This enables evaluation of dynamic processes such as epidermal architectural changes or inflammatory infiltrates.

Recent studies have assessed the applicability of RCM to evaluate the response of actinic keratoses to treatment with imiquimod or 5-aminolevulinic acid and photodynamic therapy, as well as of basal cell carcinomas treated with imiquimod. To date, RCM has accurately established the presence of BCC before treatment and its responsiveness to the treatment regimen with imiquimod^{30,31}. A more recent report has evaluated the use of RCM for the evolution of BCC treated with imiquimod and photodynamic therapy; and also correlated the RCM findings with FCM (fluorescence confocal microscopy)⁵⁷.

These studies may be also useful in order to understand the physiopathological mechanisms (inflammatory response, microvascular changes, tissue restitution, etc.) involving these novel non invasive therapies.

Conclusion and Future Perspectives

Despite its limitations in terms of probing depth and attachment to the skin, reflectance-mode confocal microscopy represents the beginning of a new era for dermatology, particularly oncologic dermatology. In research, it offers advantages both *ex vivo* and *in vivo* over conventional histology. RCM permits examination of samples without previous manipulation of the tissue, which may hinder further testing of the same sample. *In vivo*, RCM can be used to study normal processes or physiopathologic processes non-invasively over time. It can also be used to establish the presence or absence of reactive immunologic events; to guide tissue sampling including fine needle aspiration of cells for molecular analysis (DNA, RNA or proteins) or detection of specific immunological responses against neoplastic cells; finally, RCM can be used to establish timing and dosing responses to non-invasive therapies such as cryotherapy, imiquimod, or photodynamic therapy.

Clinically, RCM has value both *ex vivo* and *in vivo*. Biopsies can first be evaluated using RCM, using conventional histology to confirm or refute the RCM findings on the same sample. However, it is *in vivo* where RCM exhibits its maximum potential, being useful for *in vivo* diagnosis, assessment of the boundaries of the lesion pre- or post-surgery; and monitoring response to non-invasive therapies.

The year 2008 saw the formation of an international RCM group (www.skinconfocalmicroscopy.org), constituted by basic researchers, clinicians using RCM for diag-

nosis and monitoring and experts related to different aspects of the application of RCM to dermatology. The aim of this group is to spread the use of RCM in dermatology, providing a forum for free communication of results and the establishment of meaningful collaborations, as well as to educate potential new users in the power of this technology.

Despite the plethora of applications already described, RCM is just starting to deliver its promise, and further investigations will determine its real potential in the future management of skin cancer.

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Conflict of interest

Dr. González is investigator of a NCI-funded grant to Lucid, Inc.

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