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In Vivo Confocal Microscopy: A Promising Diagnostic Method for Cutaneous Oncology

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Abstract

Confocal microscopy enables the acquisition of non-invasive microscopic images with real-time visualization of nuclear and cellular morphologies *in vivo,* which is analogous to histological imaging at high magnification. Thus, this tool has become useful in the diagnosis of melanoma and non-melanoma cutaneous tumors. The resulting images are oriented parallel to the surface of the skin (transverse sections), which is the major difference compared to conventional histology. The fact that confocal microscopy images the tissue in the horizontal plane, similar to dermoscopy, and provides high magnification with a cellular resolution that is similar to that of histopathology, makes this technique an important area of clinical research in evaluating skin cancer. The objective of the article is to describe this technique and its primary diagnostic findings for different types of skin cancer.

Keywords: Confocal microscopy; Histopathology; Dermoscopy; Melanoma; Non-melanocytic skin tumors

Introduction

Confocal Microscopy (CM) is a technology that allows noninvasive acquisition of microscopic images with real-time visualization of micro-anatomic structures (cells, nuclei, and tissue architecture) in the skin at a resolution similar to that of pathology [1,2].

The first reported use of confocal microscopy in human skin *in vivo* was published in 1995 [2]. Since then, this technique has been improved, and there are numerous scientific publications regarding its applications in cutaneous oncology. Many studies have suggested the potential indications for dermatology.

The images obtained using this method is in planes parallel to the skin, similar to dermoscopic images but different from conventional histological sections. Thus, correlating these two methods is challenging [3-5]. A basic understanding of the features of normal skin, as well as a thorough knowledge of dermoscopy and its histopathological significance, is helpful in the interpretation of the confocal images because the criteria described in CM often corresponds directly to the dermoscopic features in the same plane [6].

Furthermore, CM shows promise for the clinical diagnosis of pigmented skin lesions when one is unable to arrive at a diagnosis using dermoscopy. CM also has great potential for increasing the diagnostic accuracy of melanocytic lesions. Pellacani et al. [7] and Guitera et al. [8] recently demonstrated that, in the diagnosis of melanoma, the use of CM by specialists resulted in a sensitivity that is comparable to dermoscopy (approximately 90%) while doubling the specificity (70% for CM and 32% for dermoscopy). The difference in specificity was even more pronounced in favor of CM when evaluating slightly pigmented lesions and pink lesions that do not generally produce specific dermoscopic findings [9].

Confocal Microscopy and Histopathology

In vivo CM offers some advantages as compared to conventional histology. The examination is painless, noninvasive and does not produce tissue damage. The skin is not altered by fixation or staining, minimizing artifacts and disruptions in the original tissue structure. The data from this real-time exam are obtained more quickly than from routine histology, and the same skin area can be repeatedly examined over time to assess dynamic changes, such as tissue growth, wound healing, lesion progression, and therapeutic responses [10-14]. This is one of the primary advantages of this technique when compared to conventional histology, which only reveals information about the tissue at the time the sample was collected.

The major limitation of CM is its low penetration through the dermis, reaching a maximum depth of 250-300 μm. This hinders the visualization of structures in the lower reticular dermis and hypodermis [10].

There are some important differences between CM and conventional histopathology. CM provides black-and-white images instead of the pink and purple that is seen in histological sections stained with hematoxylin and eosin. There is also a contrast inversion compared to routine histological staining with hematoxylin and eosin, where the structures that absorb the dye appear darker against the lighter background. In CM, the background is darker, and the cells appear brighter. Finally, the lateral resolution and optical section of CM are similar to those of histopathology. However, the quality of the image deteriorates as deeper structures are evaluated.

The images obtained during a CM examination are in the horizontal plane, whereas histopathological images are in perpendicular planes. This complicates the interpretation of confocal microscopy findings.

Conducting the Examination and Physical Principles

The presently available commercial confocal microscope

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(Vivascope 1500, Lucid Inc., Rochester NY) consists of an optical unit that is coupled to an articulated arm that facilitates its positioning on the patient. For the examination, a skin lesion of interest is selected, and a metal ring is attached to the skin using a transparent adhesive plastic disc. An oil interface is placed between the ring and the skin. The optical unit of the apparatus is coupled to the ring, allowing stable contact between the objective lens and the skin. A gel with a refractive index close to that of water is placed between the ring and the objective lens, which is located at the end of the articulated arm.

The laser light focuses on a small area on the lesion of interest and is reflected and scattered due to variations in the refractive index of the organelles, tissue structures and tissue elements with the same light source wavelength. This results in confocal image contrast (images in black and white). Using a near-infrared laser wavelength (diode laser with 830 nm), the melanosomes and melanin produce intense refraction and dispersion. Thus, melanocytes and basal keratinocytes, which are rich in melanin and melanosomes, respectively, are extremely bright [15,16].

The reflected and scattered light travels back through the objective lens and reaches the selective aperture (pinhole) that prevents the passage of out-of-focus light. The confocality of the microscope arises mainly from the diameter of the selective aperture (pinhole), which determines the axial resolution (i.e., the smaller the aperture, the higher the image quality) [17-20].

The confocal microscope uses a water-immersion objective lens with 30X magnification and a 0.9-NA aperture, and the image has a field of view of 0.5 × 0.5 mm. Whole-field images can be captured and stored. The fields are captured sequentially to form an overall image (mosaic). Up to 16 16 images can be grouped with the equipment currently available, providing a 8 × 8 mm field of view, which is equivalent to a 2X magnification [15,16].

The confocal microscope can also produce vertical images (Z stacks) by capturing a sequence of images in depth, starting from the surface. Vertical images can be animated, simulating real-time *in vivo* imaging. Videos can be produced to document dynamic events, such as leukocyte trafficking and blood flow [15].

Confocal Microscopy of Normal Skin

In order to diagnose skin disease, it is important to understand the normal anatomy of the skin. The appearance of normal skin in CM depends on several factors, including the skin phototype, anatomical site, sun exposure, age, and physiological conditions [21].

The stratum corneum (corneal layer) consists of large, bright, and anucleate polygonal cells (10-30 µm) with dark fissures and ridges (dermatoglyphics) (Figure 1A). The stratum granulosum (granular layer) is located 15-20 µm under the stratum corneum and has large bright cells (25-35 µm) with a granular cytoplasm and large, dark, and oval-shaped nuclei (Figure 1B). The stratum spinosum (spinous layer) has a "honeycomb" pattern with polygonal cells (10-25 µm), dark nuclei, and a bright cytoplasm (Figure 1C) and is located 20-100 µm below the

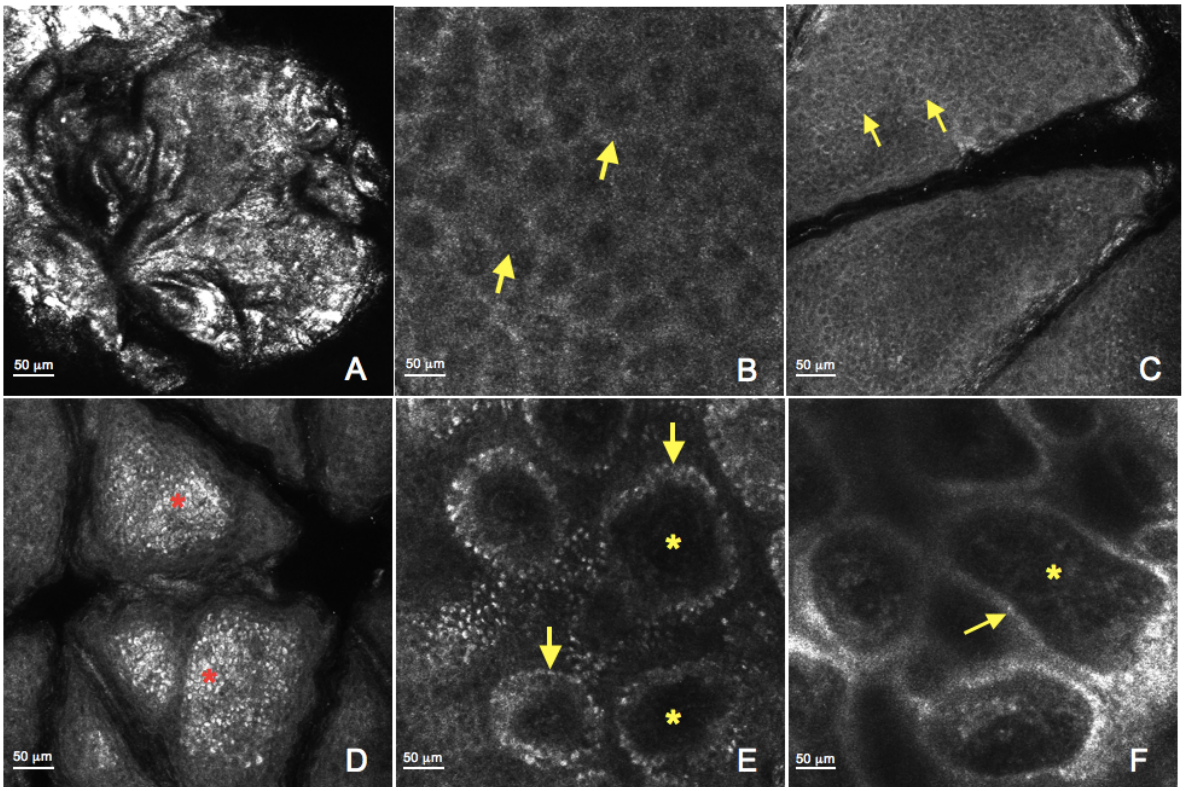

Figure 1: CM images (0.5 x 0.5 mm) of normal skin. (A) Stratum corneum appears as highly refractile surface with dark skin folds. (B) Stratum granulosum has a regular honeycomb pattern and shows polygonal keratinocytes with grainy cytoplasms and dark central nuclei (arrows). (C) Stratum spinosum has a honeycomb pattern and spinous keratinocytes are smaller with dark central nuclei surrounded by a rim of bright cytoplasm (arrows). (D) Stratum basalis consists of a cobblestone pattern formed by clusters of small bright cells usually without nuclei visualized (asterisks). (E) Dermoepidermal junction consists of dermal papillary rings (arrows) surrounding dermal papillae (asterisks). (F) Papillary dermis consists of a network of reticulated grey fibers (asterisk), with central capillary vessels surrounded by bright cellular rings (arrows) correlated with basal keratinocytes.

stratum corneum. The stratum basalis (basal layer) consists of smaller bright keratinocytes containing melanin, which typically accumulates as a supranuclear cap and assumes a "cobblestone" appearance (Figure 1D). At the dermal-epidermal junction, melanocytes and basal keratinocytes (small, 7 to 12 µm cells) are round and form bright rings of cells (riniform pattern) around a dark area (the papillary dermis with its central vessels) (Figure 1E). In the papillary dermis, reticulated collagen fibers or bundles and melanophages surround small vessels (Figure 1F). Melanophages appear as large cells, with defined and bright edges in the upper dermis. The reticular dermis has a reticulated pattern consisting of collagen bundles and elastic fibers (Table 1) [2,15,22].

Confocal Microscopy of Non-Melanocytic Skin Tumors

The characterization of skin cancer using confocal microscopy is an important area of clinical research with the potential for making noninvasive diagnoses and managing skin cancer. The advent of less invasive topical therapies, such as imiquimod and photodynamic therapy, would benefit the development of noninvasive diagnostic methods to identify the tumor type and its surgical margins, and monitor its therapeutic response [23-26].

Squamous Cell Carcinoma

CM findings that are consistent with Squamous Cell Carcinoma (SCC) include irregular hyperkeratosis with parakeratosis, architectural disarray affecting the entire thickness of the epidermis, and enlarged nuclei with pleomorphic keratinocytes in the granular and spinous layers [23]. Rishpon et al. found the presence of epidermal architectural disarray (atypical honeycomb pattern), round nucleated cells in the granular and spinous layers, and round blood vessels across the dermal papilla as key findings for the SCC diagnosis using CM (Figure 2) [27]. The limitations in visualizing the dermis do not allow the differential diagnosis of superficially invasive squamous cell carcinoma vs. *in situ* squamous cell carcinoma (Bowen's disease) (Table 2).

Basal Cell Carcinoma

Basal Cell Carcinoma (BCC) is the most common skin cancer in humans. Reflectance Confocal Microscopy (RCM) can augment dermoscopic diagnosis, allowing the clinician to diagnose BCC more confidently in real-time. Most RCM features of BCC appear at the level of the superficial dermis or Dermal-Epidermal Junction (DEJ). In the mosaic images, akin to low-power magnification microscopy, bright tumor islands can be seen, and are often well defined by surrounding cleft-like dark spaces from the surrounding dermis (Figure 3). Bright tumor islands are typically seen in pigmented BCCs. The presence

EPIDERMIS	
Corneal layer	Bright images of polygonal anucleated corneocytes (10-30 µm), grouped in "islands" separated by skin folds (dark areas)
Granular layer	Presence of 2-4 cell layers (25-35 µm) with dark, central, and oval-shaped nuclei, surrounded by bright granular cytoplasm
Spinous layer	A "honeycomb" appearance with smaller cells (15-25 µm) and well-demarcated limits
Basal layer	Group of bright cells measuring 7-10 µm. At the dermal-epidermal junction, rings of bright basal cells surrounding a darker central area represented by the dermal papilla (edged papilla)
DERMIS	
Papillary dermis	Gray reticulated fibers with central blood capillaries surrounded by rings of bright basal cells (edged papillae)
Reticular dermis	Reticulated pattern (bands of collagen and elastic fibers)

Table 1: CM findings for normal skin.

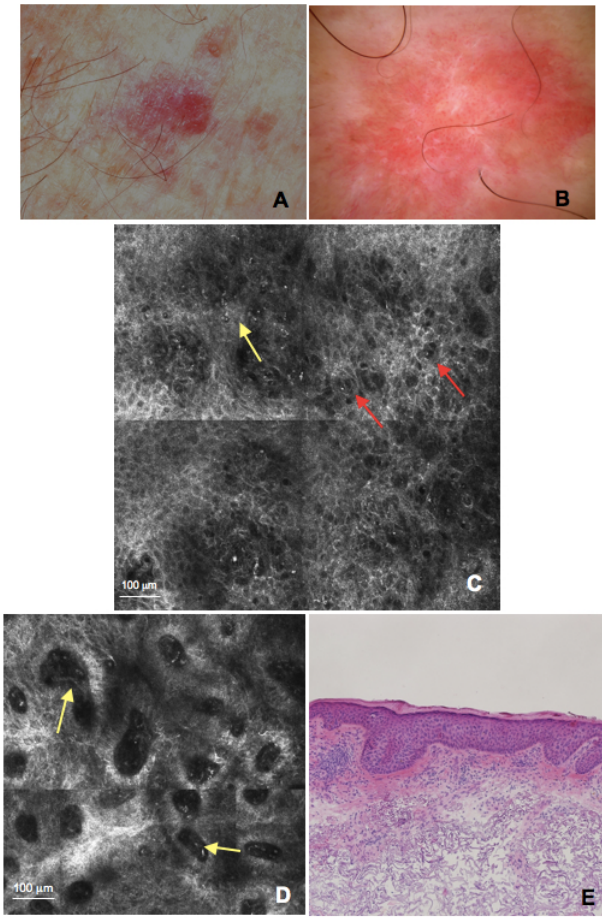

Figure 2: Clinical photograph of a red scaly plaque of the arm (A). This lesion shows on dermoscopy (B) features suggestive of SCC such as vascular pattern characterized by vessels appearing as red dots. CM mosaic image (C, 1 x 1 mm) at the level of the spinous granular layer shows atypical honeycomb pattern with pleomorphic cells and broadening of the intercellular spaces (red arrows), and small bright cells suggestive of inflammatory cells (yellow arrow). CM image (D, 0,75 x 0,75 mm) at the level of the DEJ shows dilated, round blood vessels within dermal papillae (yellow arrows). On histopathology (E), the lesion proved to be a SCC *in situ*.

of tumor islands as hyporeflective areas ("dark silhouettes") that are darker than the surrounding dermis or basal epidermis are mostly seen in non pigmented BCCs. However, the dense stromal collagen around the neoplastic aggregates creates a distinct imprint on mosaic images of dark silhouettes outlined by collagen bundles at the level of the superficial dermis [9]. The bright tumor islands and the dark silhouettes seen on RCM correlate on histopathology with aggregates of basaloid cells with peripheral palisading of nuclei (Figure 3). The cleft-like dark spaces around tumor islands is an interesting *in vivo* RCM observation, since the cleft seen around the neoplastic aggregates on histopathology is considered an artifact of tissue processing. The clefting on RCM probably corresponds to mucin surrounding the neoplastic aggregates.

At individual RCM images, akin to higher power microscopy, within the tumor islands, elongated nuclei can sometimes be discerned as exhibiting peripheral palisading of nuclei. The adjacent dermal stroma contains plump-bright oval to stellate cells with indistinct borders compatible with melanophages, or small bright dots compatible with smaller inflammatory cells such as lymphocytes. Dilated blood vessels coursing in the *en-face* plane of imaging can be seen in real-time or

Squamous cell carcinoma	Irregular hyperkeratosis Epidermal pleomorphism and nuclear enlargement Architectural disarrangement throughout the epidermis (atypical "honeycomb" pattern) Round nucleated cells in the granular and spinous layers Round blood vessels throughout the dermal papilla
Basal cell carcinoma	Orientation of the tumor cells nuclei along the same axis ("polarization") Tumor silhouettes / Bright tumor islands Cleft-like dark spaces Dendritic structures within the tumor islands or in the overlying epidermis Plump-bright non nucleated cells Canalicular vessels

Table 2: CM findings for non-melanocytic skin tumors.

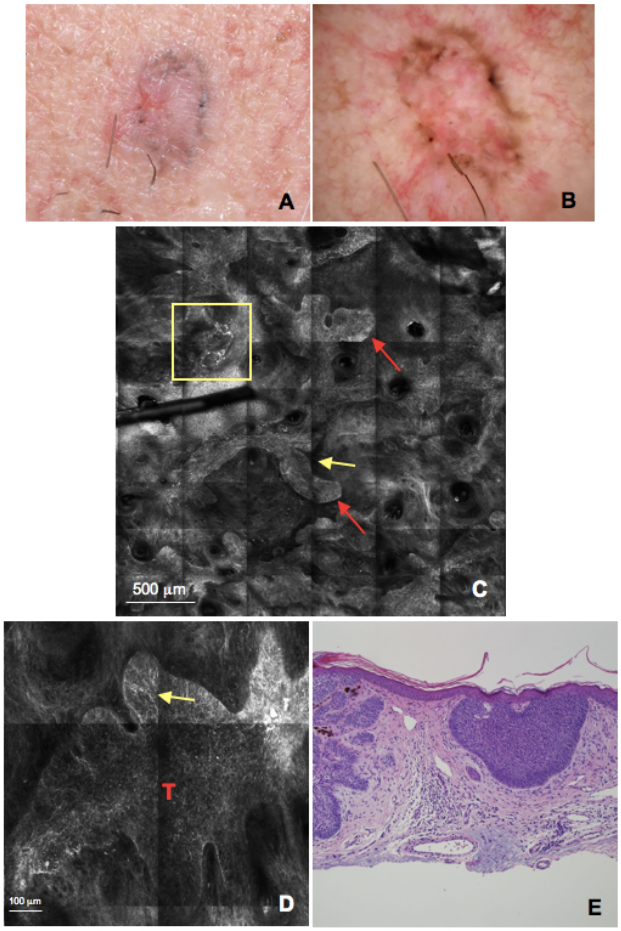

Figure 3: Clinical photograph of a pink brown papule of the left jawline (A). This lesion shows on dermoscopy (B) features suggestive of BCC such as the absence of a pigmented network and the presence of leaf-like structures, pink-white shiny areas and brown gray dots in focus. CM mosaic image (C, 3 x 3 mm) at the level of the dermoepidermal junction shows polypoid nodules of tumor cells (red arrows) surrounded by cleft spaces (yellow arrow), and bright round cells (yellow square). CM image (D, 1 x 1 mm) at the level of the DEJ shows dendritic cells (yellow arrow) at the periphery of the tumor island (T). On histopathology (E), the lesion proved to be a basal cell carcinoma.

video-mode RCM imaging, juxtaposed to the tumor islands [9,28,29]; these blood vessels often exhibit rolling of leukocytes. At times, multiple bright dendritic structures and dendritic cells can be seen within the tumor islands (Figure 3); these dendritic cells within neoplastic aggregates of BCC have been shown by immunohistochemistry to be melanocytes [30]. Interestingly, concomitant dendritic cells in the overlying spinous layer of the epidermis have been shown to correlate with Langerhans cells [31]. Elongated nuclei, polarization of nuclei and a pleomorphic overlying epidermis have also been previously described as features of BCC (Table 2) [28,29].

A multicenter retrospective study of 152 lesions [29] showed that the presence of at least two of these criteria resulted in a sensitivity of 100% for the diagnosis of BCC. As the number of criteria was higher, the specificity increased, so that when at least four of the criteria were present, the specificity was 95.7%, which provided the best agreement between the high sensitivity and the high specificity. The single criterion for the most sensitivity and specificity was the presence of polarized nuclei (91.6% and 97%, respectively). These results showed little variability according to BCC location and subtypes. CM can also be useful in monitoring therapeutic response after the use of noninvasive therapies, such as topical imiquimod and photodynamic therapy [24,26].

Confocal Microscopy of Melanoma

Cutaneous melanoma is the most aggressive skin cancer in humans, and its diagnosis can often be challenging even for the best clinicians.

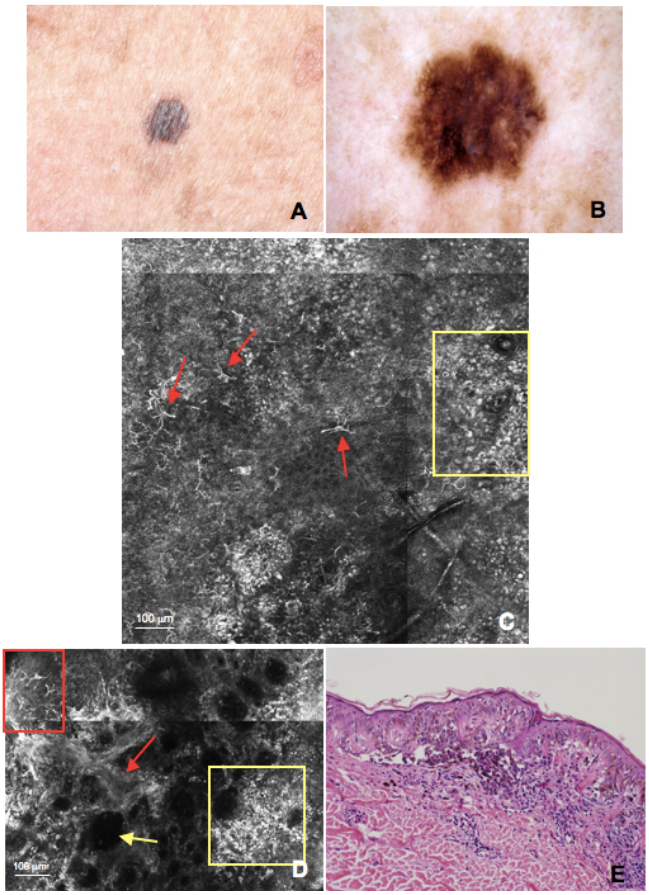

Figure 4: Clinical photograph of a prominent mole on the back (A). This lesion shows on dermoscopy (B) multiple colors, atypical network and gray dots/granules. CM mosaic image (C, 1 x 1 mm) at the level of the suprabasal layer shows spindle shaped cells (red arrows) and nucleated cells (yellow rectangle) infiltrating the epidermis. CM image (D, 0,75 x 1 mm) at the level of the DEJ shows junctional thickening (red arrow), non-edged papillae (yellow arrow), numerous pagetoid cells (yellow square) and dendrites (red rectangle). On histopathology (E), the lesion proved to be a melanoma.

The clinical and dermoscopic examination have accuracy up to 85% for experienced clinicians. The ability to recognize melanocytes and analyze their morphology and distribution is the basis for the use of *in vivo* CM in diagnosing melanomas [4,6,7,32-34].

CM of a melanoma reveals enlarged, pleomorphic cells (oval, fusiform, or stellate in shape) with branching dendrites and large, dark, and eccentrically arranged nuclei. These cells can be identified in the superficial layers of the epidermis (pagetoid dissemination) and the dermis (Figure 4). Disruptions in the regular "honeycomb" architecture of the spinous layer are also common, with poorly defined cell borders and bright grainy particles, which probably represent the melanin distributed throughout the epidermis ("melanin dust") [6]. The dendrites in the melanoma lesions are thick and bright. The dermal papilla may have an irregular shape or size, may be poorly demarcated without a bright ring of basal cells (non-edged papillae), or may be completely unclear due to a flattening of the dermal-epidermal junction (Figure 4). Cerebriform clusters or nests of scattered cells can occasionally be identified in the dermis. These structures represent clusters of irregular bright cells with undefined contours, pleomorphism and surrounded by "melanin dust".

The criteria for distinguishing between benign and malignant pigmented lesions are currently being described. Nevertheless, CM images of the dermal-epidermal junction enable the discrimination between nevi and melanomas with a high degree of certainty by evaluating of the overall architecture of the lesion and its melanocytic cytomorphology [7,8].

A diagnostic algorithm using CM to evaluate dermoscopically and clinically equivocal melanocytic lesions was recently proposed by Pellacani et al. [35]. This algorithm has two major and four minor criteria associated with malignancy and a total score ranging between 0 and 8. The major criteria (scored 2 points each) are poorly delineated papillae (non-edged papillae) and atypical melanocytes in the basal layer. The minor criteria (scored 1 point each) include pagetoid cells (round, bright, and nucleated cells that correspond to melanocytes) in the epidermis above the basal layer, the disseminated infiltration of pagetoid cells throughout the lesion, cerebriform clusters in the dermis (confluent clusters of cerebriform cells with low reflectivity), and bright nucleated cells within the dermal papilla (corresponding to isolated melanocytes in the dermis) (Table 3). A score ≥ 3 suggests a diagnosis of melanoma with 97.3% sensitivity and 72.3% specificity [35]. Moreover, Gerger et al. described another important criterion for the melanoma diagnosis: the loss of boundaries between keratinocytes [36].

Conclusion

For a trained physician, CM is a sensitive and specific tool for the early detection of melanomas and other skin tumors. When examinations are methodically performed using the diagnostic algorithms, CM provides comprehensive architectural and cytoarchitectural assessments of the epidermis, dermal-epidermal junction, and upper dermis. Cellular atypia and pleomorphisms, including certain nuclear morphologies, can be visualized *in vivo* to assist in the diagnosis.

Currently, performing a diagnosis using CM has some challenges. First, the image depth is limited, impairing the assessment of the deep dermis and hyperkeratotic lesions. Second, the cellular resolution is suboptimal, and the cellular details indispensable in histopathological evaluation of lesions are difficult to analyze using CM. Third, *in vivo* images preclude the use of chemical dyes that are extremely useful in the histopathological identification of specific cell types.

Melanoma	Keratinocyte architectural disarray (loss of the epidermis "honeycomb" pattern) Non-edged papillae Cellular atypia Round or dendritic (pagetoid) cells in the epidermis Cerebriform nests in the papillary dermis Nucleated cells in the dermal papilla

Table 3: CM diagnostic criteria for melanoma.

However, one CM additional advantage is the possibility of correlating its findings with those provided by dermoscopy, improving both the sensitivity and specificity of the diagnosis and the correlation between these two noninvasive methods. At the same time, CM may increase the specificity for establishing diagnoses in areas where dermoscopy shows less conclusive findings [5].

CM offers great potential for the management of skin cancer, particularly cutaneous melanoma. The ability to noninvasively diagnose a skin tumor, determine its margins, and provide its follow-up makes this technique a useful tool in clinical dermatology. Furthermore, CM enables the study of the pathophysiological processes in real time with no tissue damage and by the single technique over time.

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Conflict of Interest

The authors state no conflict of interest.

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