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Abstract. Confocal laser scanning microscopy (CLSM) represents an emerging technique for the noninvasive histomorphological analysis of skin *in vivo* and has shown its applicability for dermatological research as well as its value as an adjunct tool in the clinical management of skin cancer patients. Herein, we aim to give an overview on the current clinical indications for CLSM in dermatology and also highlight the diverse applications of CLSM in dermatological research. [DOI: 10.1117/1.JBO.18.6.061212]

Keywords: cosmetics; fluorescence confocal microscopy; reflectance confocal microscopy; skin aging; skin cancer.

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1 Introduction

Confocal laser scanning microscopy (CLSM) represents an emerging technique for the noninvasive histomorphological analysis of skin *in vivo*. In the past decade, various studies have shown the applicability of *in vivo* reflectance CLSM for the noninvasive diagnosis of melanocytic skin lesions as well as nonmelanoma skin cancer. Furthermore, CLSM has been used for the evaluation of benign inflammatory skin diseases.

Recently, novel devices combining the standard confocal laser scanning microscopy in reflectance mode with multi-laser fluorescence techniques have been used for clinical studies.[1]

In addition, *ex vivo* set-ups allow for the evaluation of biopsy specimens enabling rapid evaluation of tumor margins in Mohs' micrographic surgery.

Herein, we aim to give an overview of established CLSM techniques for *in vivo* diagnosis of skin disease, including a brief outlook on emerging CLSM systems currently under investigation.

2 History and Technical Principle of CLSM

In 1955, Marvin Minsky described the optical principle of confocal laser scanning microscopy at Harvard University in Boston, Massachusetts.[2] However, this innovative technology was only published as a patent and remained more or less unrecognized until the 1990s, when Rajadhyaksha et al. first conceived reflectance mode CLSM for skin imaging in its current design.[3] Since then, continued technical innovations decreased the size of the machine, thus facilitating the imaging process. Nowadays, handheld devices are available, which can be used for rapid *in vivo* examination, facilitating evaluations in difficult anatomic areas and also at the bedside.

The technical principle of CLSM is based on the illumination of a small spot within the tissue using a point light source. Reflected light is guided through a pinhole in front of the detector. Thus, only reflected light from the tissue spot, which is in-focus, is used for image generation, whereas light coming from out-of focus planes is eliminated. Horizontal movement of the objective obtains parallel sections of the skin. Endogeneous chromophores in the skin are responsible for the differences in reflectivity, which results in gray scale images. Of these, melanin represents the skin structure with the highest refractive index and therefore provides the highest contrast by CLSM. On CLSM images, melanin-containing cells (both pigmented keratinocytes and melanocytes) appear white. But also other skin structures such as keratin, collagen, or haemoglobin are natural contrast agents.

3 Application of *In Vivo* CLSM for Skin Cancer Diagnosis

Early diagnosis of skin cancer represents the most important factor in the management of the increasing skin cancer burden and may significantly reduce mortality and morbidity of the patients. Most skin cancers can be cured by simple excision when diagnosed at an early evolving stage. This particularly applies to melanoma as its prognosis is directly correlated with anatomic depth of invasion.[4] However, also in nonmelanoma skin cancers (NMSC), such as basal cell carcinoma and squamous cell carcinoma, early diagnosis is essential for positive therapeutic outcome, prognosis, and cosmesis. Although these tumors rarely metastasize, they are often located in cosmetic sensitive areas of the face and scalp where larger excisions may give rise to disfiguring scars and healing impairment. In the past decade, a number of noninvasive treatment modalities have become available, thus optimizing the therapeutic management for selected subtypes of NMSC. In that regard, it has been suggested that noninvasive diagnostic techniques such as *in vivo* CLSM may be applied for therapeutic monitoring and follow-up.

Despite the great potential of CLSM, it also has limitations, mainly including the restricted penetration depth. Furthermore, the evaluation of a skin lesion by CLSM requires 5 to 10 min and is therefore more time consuming than clinical evaluation or dermoscopy alone. Due to the horizontal images of CLSM, early invasion of skin tumors may be very difficult to diagnose as the basement membrane cannot be visualized. Lastly, the interpretation of CLSM images requires training and experience

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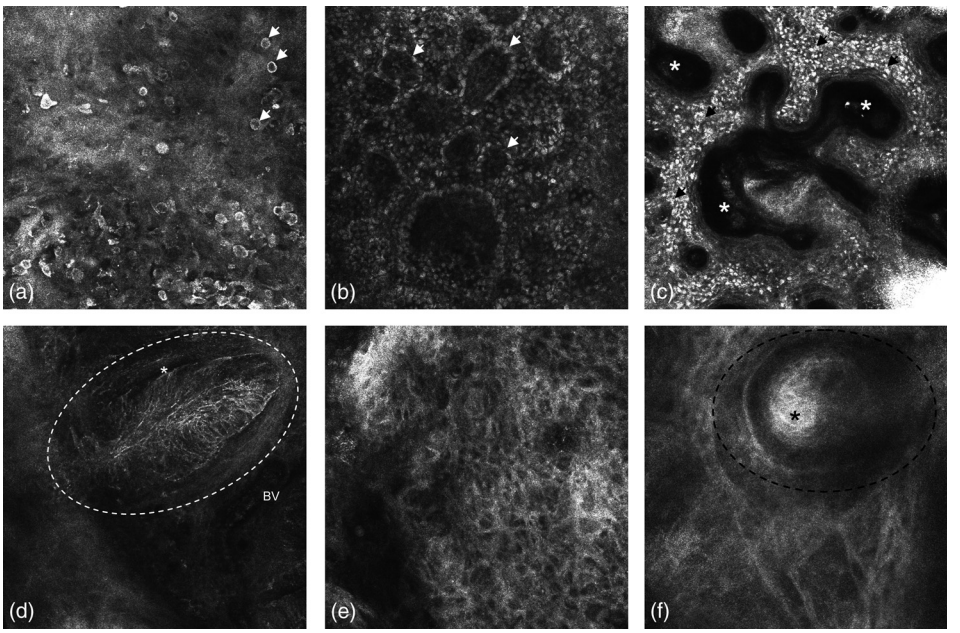

Fig. 1 Representative RCM images (500 × 500 μm) illustrating specific patterns of different skin tumors. (a) An RCM image of a melanoma with large atypical nucleated cells (black arrows) in the epidermis, representing widespread pagetoid spread of atypical melanocytes. (b) An RCM image of a benign nevus with the presence of small round cells of bright reflectance at the dermo-epidermal junction. A ringed pattern with well-edged papillae (white arrows) and a cobble stone pattern of the overlying epidermis can be noted. (c) The typical cerebriform pattern of the epidermis in seborrheic keratosis. Areas of epidermis with pigmented keratinocytes (black arrows) and invaginations filled with keratin can be noted (white asterisks). (d) A typical pattern of a basal cell carcinoma with the presence of tumor islands (white dashed circle) consisting of elongated cells with palisading in the periphery. Between the BCC tumor islands and the surrounding fibrous stroma small cleft-like spaces (white asterisk) can be noted that correspond to mucin deposition. Dilated blood vessels (BV) can be seen in the surrounding stroma. (e) Illustrates the atypical honeycomb pattern of an actinic keratosis, different size and shape of keratinocytes can be noted (pleomorphism). (f) Shows an RCM image of an invasive squamous cell carcinoma illustrating tumor islands with central bright center (black dashed circle) located in the upper dermis corresponding to keratinization (black asterisk).

of the observer as it is the case with every diagnostic technique (Fig. 1).

3.1 *Melanoma*

Different types of melanoma can be distinguished, including superficial spreading melanoma (SSM), lentigo maligna melanoma (LMM), nodular melanoma (NM), and acrolentiginous melanoma (ALM). Of these, SSM represents the most common type, accounting for about 70% of all melanomas diagnosed. NM is the second most common form and diagnosis is often challenging as the tumor arises on normal skin and clinical algorithms like the "ABCD" rule usually fail to obtain the diagnosis in early stages. Furthermore, the tumors lack a horizontal growth phase and vertical spread occurs at an early stage, resulting in higher metastatic potential. In contrast, LMM occurs on chronically sun-exposed skin of the elderly often showing a prolonged horizontal growth phase. However, clinical as well as histopathological diagnosis of LMM may be difficult to obtain in the background of actinic damage with the presence of multiple solar lentigines. As *in vivo* CLSM offers the possibilty of evaluating skin tumors noninvasively and with near-histological resolution, the use of CLSM for melanoma and its differential diagnosis has been the subject of extensive research in this field. Earlier studies have focused on the correlation of CLSM features of melanoma and naevi with histology[5–8] and showed the reproducibility of histological criteria such as pagetoid spread and typical nests of melanocytes by RCM. Since then, multiple studies have evaluated the applicability of RCM for the diagnosis of melanoma and equivocal pigmented lesions[9–13] and it has been shown by Pellacani et al. that CLSM may improve the diagnostic specificity and may thus reduce the number of unnecessary biopsies of benign lesions. In 2007, Pellacani et al. described the RCM features of epidermal disarray, pagetoid cells in the epidermis, nonedged papillae, cellular atypia at the junction, atypical nests, and bright nucleated cells in the dermis as being associated with melanoma.[9] Based on these criteria, a score was developed that included two major criteria, each scored 2 points (nonedged papillae, cellular atypia), and 4 minor criteria, each scored one point (roundish pagetoid cells, widespread pagetoid infiltration, cerebriform nests and nucleated cells in the dermis). A total RCM score $\geq$3 showed a sensitivity of 91.6% and a specificity of 69.3% for the diagnosis of melanoma. Furthermore, the two step method was proposed by Segura et al.[14] The two-step method described four features that distinguished melanocytic lesions from nonmelanocytic lesions including cobblestone pattern of epidermal layers, pagetoid spread, mesh appearance of the dermoepidermal junction, and the presence of dermal nests as criteria for melanocytic lesions. In the second step of the algorithm, two protective criteria were identified (typical basal cells, edged papillae) and scored minus one and two high risk criteria (roundish pagetoid cells, atypical nucleated dermal cells) were scored plus one. A lesion with total score of 0 to 2 was indicative to be most probable melanoma, with a sensitivity and specificity of 86.1% and 95.3%, respectively. Very recently, a large study on 710 consecutive cases analyzed the sensitivity and specificity of two algorithms for the diagnosis of basal cell carcinoma (BCC)

and melanoma (MM).[15] In this study, the diagnostic accuracy for the BCC algorithm was 100% sensitivity and 88.5% specificity. The MM algorithm showed a sensitivity of 87.6% and a specificity of 70.8%. Interestingly, the four melanomas that were misdiagnosed by the RCM method were of nevoid subtype on histopathological examination. In conclusion, RCM is applicable for the diagnosis of melanoma, showing good sensitivity and specificity values. However, rare subtypes of melanoma (nevoid melanoma, spitzoid melanoma) represent a potential pitfall that needs to be considered. Careful clinical and dermoscopy correlation of the lesion is beneficial and doubtful cases need to be excised for histopathological examination [Fig. 2(a)].

3.2 *Basal Cell Carcinoma*

Basal cell carcinoma (BCC) represents the most common skin cancer in humans and typically arises on chronically sun-exposed skin. A number of different subtypes can be distinguished that vary regarding the clinical presentation and include among others nodular, superficial, pigmented, and sclerosing type of BCC. On histological examination BCCs are characterized by tumor aggregates consisting of basophilic cells that typically show a rim of elongated palisading cells in the periphery and that are separated from the surrounding stroma by clefts. *In vivo* RCM has early been used to investigate features of basal cell carcinoma[16,17] and in 2004 a multicenter study evaluated the sensitivity and specificity of five independent parameters, including architectural alteration and cellular pleomorphism of the overlying epidermis, areas of refratile tumor cells with longated, monomorphic nuclei, nuclear polarization, increased dermal vasulature, and prominent inflammatory infiltrate.[18] In this study, the identification of two or more criteria showed a sensitivity for BCC diagnosis of 100%, whereas the presence of four or more criteria showed a sensitivity of 82.9% and a specificity of 95.7%. Further studies have described RCM features of pigmented BCC that includes the additional presence of dendritic cells within tumor nodules of BCC[19] and have analyzed the applicability of RCM for monitoring of treatment response to imiquimod and cryotherapy as well as the applicability of RCM as an adjunct tool to Moh's micrographic surgery.[20,21] As already mentioned above, a recent study confirmed the high diagnostic accuracy of RCM for the diagnosis of BCC, showing a 100% sensitivity and 88.5% specificity for the proposed BCC algorithm by Guitera et al.[15]

4 Actinic Keratosis, Bowen's Disease, and Invasive Squamous Cell Carcinoma

Actinic keratosis (AK), Bowen's disease (BD), and invasive squamous cell carcinoma (SCC) represent a continuum of a disease process in actinically damaged skin. AK and BD share histological and molecular genetic features of invasive SCC, but are limited to the epidermis.[22] As both AK and BD represent superficial forms of skin cancer they seem most suitable for RCM evaluation. However, these lesions are also often characterized by the presence of hyperkeratotic scale that may impair image resolution and diagnostic accuracy. In invasive SCC, prominent hyperkeratosis may significantly interfere with the imaging of underlying structures.

A preliminary study by Aghassi et al. in 2000 already described the presence of hyperkeratosis, lower epidermal nuclear enlargement and pleomorphism, and architectural disarray as features of AK in a limited subset of patients.[23] Larger studies performed by three independent groups have shown the applicability of RCM for the diagnosis of nonhyperkeratotic AKs.[24–26] Ulrich et al. analyzed 46 AKs and reported the presence of architectural disarray and cellular pleomorphism as the best predictor for AK; sensitivity and specificity values in this study ranged from 80% to 98.6%. In the study by Ahlgrimm-Siess et al., 30 actinic keratoses were analyzed and a sensitivity of 93.34% and specificity of 88.34% could be achieved by two clinical dermatooncologists (positive predictive value 88.94%, negative predictive value 93.15%). A one-step algorithm was proposed by the authors, which was based on only one criterion (irregular keratinocyte cell borders) and allowed a correct diagnosis in 86.67% of actinic keratoses and 85% of normal skin. In a further study, Rishpon et al. analyzed 7 AKs, 25 SCCs *in situ*, 3 invasive SCCs, and 3 keratoacanthomas and described the presence of an atypical honeycomb or a disarranged pattern of the spinous-granular layer, round nucleated cells at the spinous-granular layer, and round blood vessels traversing through the dermal papilla as key RCM features of SCC. The features of architectural disarray and cellular polymorphism that has been used in earlier studies correlate to the feature of atypical honeycomb pattern that represents the most important feature for RCM diagnosis of AK. Recent studies have also evaluated RCM for monitoring of AKs during treatment and for the assessment of treatment response,[27] indicating the applicability of RCM for this indication [Fig. 2(b)].

5 Application of *In Vivo* CLSM for Dermatological Research

The noninvasive nature of CLSM allows evaluations of skin sites over time, thus enabling the investigation of dynamic physiological or pathological skin processes *in vivo* and at different timepoints. A number of investigations of normal and diseased skin *in vivo* have analyzed the reaction patterns to exogenous stimuli such as UV-radiation, contact irritants, and superficial skin wounds. Owing to the advantage of CLSM to analyze skin and its appendages in its native state, any assessment may be made with no further tissue alteration or processing.

5.1 *Contact Dermatitis*

Contact allergies are of high relevance in occupational and clinical dermatology as well as cosmetic research, such that accurate diagnosis is of utmost importance. The gold standard of diagnosis is the clinical evaluation of patch tests, however, accuracy of test results ranges from 75% to 85%, still yielding a substantial number of false positive/negative test results. Furthermore, there is a limited availability and suitability of experimental *in vitro* or *ex vivo* test kits to increase diagnostic accuracy. This has prompted further research for noninvasive, diagnostic tools for *in vivo* analysis of patch-test sites. González and his group were the first to describe the CLSM features of contact dermatitis (CD) *in vivo* and noninvasively.[28] Thereby, features such as spongiosis, vesicle formation, exocytosis, and parakeratosis have been defined and correlated with routine histology. It was shown that CLSM may aid in the distinction of allergic (ACD) and irritant contact dermatitis (ICD) *in vivo* based on features seen upon CLSM evaluation.[29] The most characteristic feature of ICD was the disruption of the corneal layer by exogenously applied irritants. Epidermal spongiosis and exocytosis on the other hand were comparable for both ACD and ICD after correcting for clinical score.

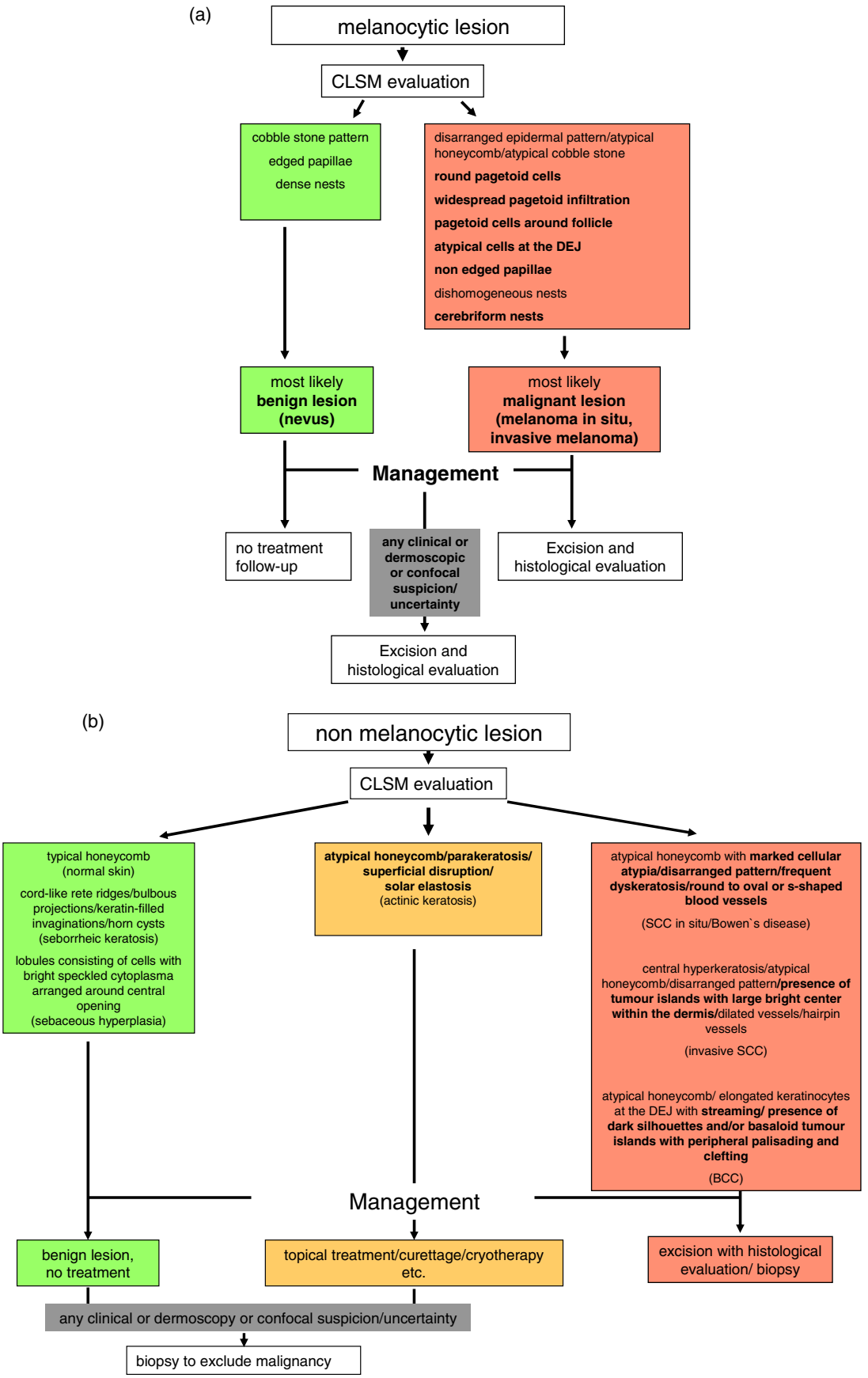

Fig. 2 (a) The CLSM criteria of melanocytic and (b) nonmelanocytic skin tumors including diagnostic and therapeutic algorithms for the most common entities.

Investigations were continued to evaluate the kinetic evolution of ACD and ICD over time.[30] The main differences included a prolonged activity of ACD compared to ICD, whereas ICD presented with features of intraepidermal necrosis as well as superficial disruption.[30] Furthermore, ethnic variability in skin response following contact with a common household irritant has been analyzed. Following exposure to increasing concentrations of a common household irritant (Ivory Soap™ dishwashing liquid) serial clinical and CLSM evaluations were performed at 24 and 48 h. Thereby, individual irritancy levels were determined for the two subgroups, being 10% for Caucasians and 25% for African-Americans. Interestingly, it was shown that CLSM was able to detect features of irritancy (i.e., parakeratosis, detachment of keratinocytes) in patients with no clinical signs of ICD. Following CLSM analysis, selected features such as spongiosis, parakeratosis, and disruption of the stratum corneum were more severe for Caucasians, indicating a higher susceptibility to irritants compared to African-Americans.[31,32] Overall these findings suggest a superior barrier function in African-Americans compared to Caucasians.

Another study performed a sensitivity/specificity analysis of CLSM compared to the gold standard, i.e., the clinical evaluation of patch tests.[33] Following the clinical evaluation of allergic patch test sites at 72 h, specificity/sensitivity values for respective CLSM features ranged from 92.6% to 100%. The highest sensitivity/specificity were determined for spongiosis and exocytosis of the granular and spinous layer. Interestingly, CLSM was able to visualize features of ACD in the absence of clinical findings, thereby detecting subclinical disease (Fig. 3).

5.2 *CLSM Application for Cosmetic Research*

With an increasing interest in the research and development of cosmetic and skin care products for the purpose of anti-aging, sun-care, and UV-protection, there is an increased need for noninvasive analysis for their efficacy, tolerabilibity, and physiological properties in human skin. Such tests involve an assessment of general toxicity, eye and skin irritancy, phototoxicity, and mutagenicity. The majority of products rely on a series of *ex vivo* test models, including artificial skin models, cell cultures, or *in vitro* assays. While their results may be reproducible, their actual applicability to live human skin has been a subject of controversial discussion. At the same time, animal studies for the purpose of cosmetic industry related research are to be avoided for ethical reasons. In 2009, the European Union (EU) agreed to initiate a near-total ban on the sale of animal-tested cosmetics throughout the EU, and to interdict all cosmetics-related animal testing. In that context it has been proposed that CLSM may be suitable for *in vivo* assessment of topographic, morphological assessment of physiological and pathological skin response to topically applied products. Since CLSM offers the opportunity to determine the kinetics of topically applied substances with an immediate assessment of local cutaneous changes, CLSM may be a useful tool for dermatological cosmetic research.[34,35]

6 Skin Aging

First investigations using CLSM for analysis of skin aging were performed by Sauermann et al. comparing volar forearm skin of young and older individuals.[36] Thereby, a significant increase in epidermal thickness was shown for the older group, associated with a significant decrease in the number of dermal papillae per area. The younger group on the other hand demonstrated an increased thickness of the granular layer, while the overall thickness of the stratum corneum showed no difference between the two groups.

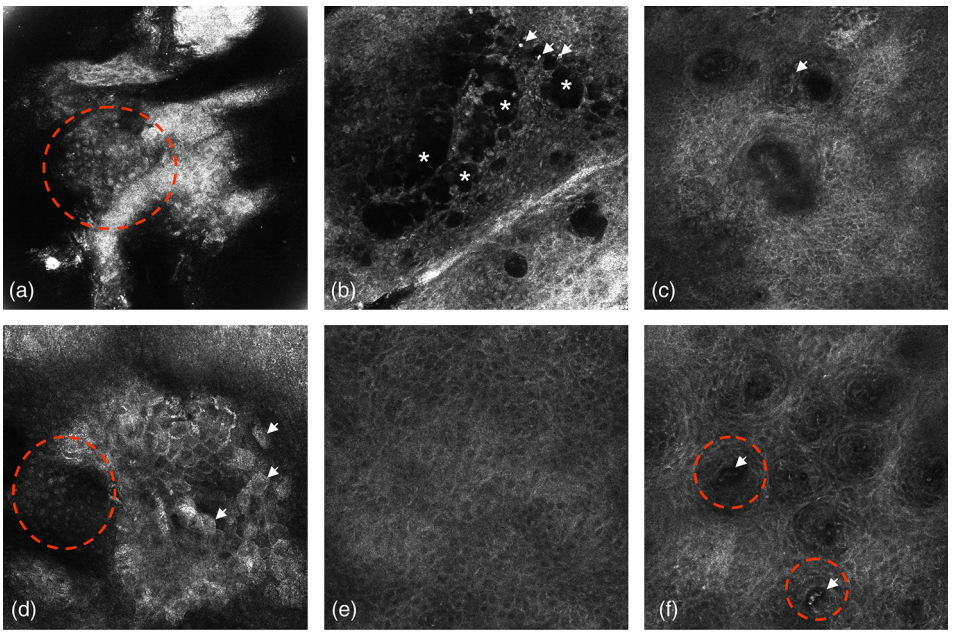

Fig. 3 Representative RCM images of (a) to (c) biopsy proven eczema and (d) to (f) psoriasis. Part (a) was obtained at the stratum corneum showing areas of parakeratosis (red dashed circle). (b) The presence of spongiotic microvesicles corresponding to dark nonrefractive spaces on RCM (white asterisk). Furthermore. small bright cells can be noted that correspond to inflammatory cells (white arrow). (c) RCM images at the DEJ with the presence of inflammatory cells (white arrow). (d) Respective images of parakeratosis (red dashed circle) and single detached corneocytes (white arrows) in a psoriatic plaque. (e) The honeycomb pattern of the epidermis. (f) Papillomatosis (red dashed circle) as present in psoriasis at the DEJ. Dilated blood vessels can be observed in the center of the papillae (white arrows).

In the same line of research, Wurm et al.[37] have performed a comprehensive analysis of chronological and photoaged skin using CLSM. They have shown, that CLSM permits a characterization and quantification of histomorphometric features of epidermal and papillary dermis as a sign of skin aging. Among 21 features analyzed, 15 were shown to be significant for quantification of skin aging. Among others, dermal papillary index (number of papillae/mm^2), the thickness of the basal layer, and an increased thickness of the granular layer permit a semiquantitative grading.[37] These changes were found to be more pronounced with increasing age, and were also associated with changes in the underlying microvasculature.

Another study by Lagarrigue et al.[38] used CLSM to quantify epidermal pigmentation and density of dermal papilla in correlation with age and skin phototype. Following an analysis of 111 healthy female volunteers, it was shown that papillary contrast at the level of the dermo-epidermal junction correlated strongly with skin pigmentation assessed clinically using the Fitzpatrick skin phototype classification. While the first studies on the detection and distribution of melanin and melanocytes have been performed as early as 2005,[39,40] these findings are also confirmatory of a preliminary study by Antoniou et al., first analyzing pigment distribution in correlation with the Fitzpatrick classification.[41] With respect to skin pigmentation, a number of studies have been aimed at analyzing the process of immediate and delayed pigment darkening reactions and their respective association with skin microvasculature.[42]

Further analyses have focused on the therapeutic effects of topically applied antioxidants such as Vit-C for skin aging.[43] These investigations have shown that five weeks of topical Vit-C application resulted in a significant increase in dermal papillary density, compared to vehicle treated control. RCM was able to show a restoration of the dermoepidermal junction in young skin, and an increase in density of capillaries within dermal papillae in aged skin compared with untreated control. These longitudinal studies illustrate the opportunity of CLSM to monitor treatment response and local skin reaction as well as dynamic events of skin repair and regeneration.

7 Cosmetic and Medical Laser Treatment

Longo et al.[44] performed a systematic analysis of local effects of epidermal and dermal changes following laser skin rejuvenation using a fractionated CO 2 laser in an ablative mode including 10 female volunteers. Following a single treatment with a radiofrequency excited ultrapulsed CO 2 laser device, four serial CLSM evaluations were performed at baseline, 3, 6, and 12 weeks after treatment. After blinded image analysis, it was shown that CLSM was able to document a disappearance of mottled pigmentation as a feature of skin dyspigmentation in photoaged skin. Furthermore, collagen remodeling was described by the appearance of newly formed collagen at week 3, seen as bright, straight fibers in parallel arrangement throughout the entire CLSM mosaic and persisting at the 12 weeks evaluation timepoint.

Xu et al. have used CLSM for monitoring and follow-up of laser treatment for infraorbital dark circles using a low-fluence Q-switched 1,064-nm laser.[45] The results of this investigation showed a dramatic decrease of melanin deposition in the upper dermis as seen by CLSM evaluation.

Other studies using laser treatment have evaluated the applicability of CLSM for follow up of cherry angioma treated by pulsed dye laser (PDL) *in vivo*.[46] Thereby, the disappearance of dilated capillary loops after PDL treatment was shown using CLSM. Aghassi et al. have also used CLSM for monitoring of PDL treatment for sebaceous hyperplasia.[47] A cohort of 10 patients with a total of 29 lesions were treated by PDL followed by serial confocal evaluations at baseline and follow-up up to eight weeks post treatment. CLSM was able to visualize the morphological and vascular aspects of dynamic treatment response, including a coagulation of blood vessels and consecutive (partial) regression of sebacious lesions.[48]

Yamashita et al. have reported on a series of subjects with benign, superficial pigmented skin lesions on the face of Asian volunteers, treated by serial sessions of intense pulsed light (IPL) therapy.[49] CLSM was used to document lesions at baseline, as well as monitor treatment response including crusting, migration of melanosomes, and overall melanocyte activity. The authors have come to the conclusion that CLSM is suitable to monitor the removal of superficial melanosomes from the basal layer, while leaving overall melanocyte activity intact.

8 Conditions with Exogenous/Endogenous Pigment Alterations/Decorative Tattoo

CLSM may be useful for evaluation of tattoo-related skin alterations. CLSM was able to visualize subepidermal deposits of dense, clustered pigment granules of up to about 3 μm in size corresponding to black tattoos, while red, blue, and green tattoos present on CLSM with a more scarce and diffuse appearance. Thereby, it was postulated that CLSM may be a potentially useful tool for evaluation of laser tattoo removal as well as monitoring skin repair after laser therapy.[34,50]

9 Melasma

Melasma is a common pigmentary facial skin disorder caused by abnormal melanin deposits. Kang et al. were the first to analyze a total of 29 patients with a clinical diagnosis of melasma using reflectance confocal microscopy,[51,52] describing a set of morphological criteria in correlation with routine histology. All patients showed a significant increase in hyperrefractile basal cells, corresponding to hyperpigmented basal keratinocytes. Selected patients showed dendritic cells in the epidermis corresponding to activated melanocytes. At the level of the dermal layer, CLSM identified plump bright cells corresponding to melanophages, a feature that has previously also been described in conjunction with melanocytic skin lesions such as benign and dysplastic nevi.

A preliminary study by Ardigo et al. included a total of ($n = 15$) patients previously diagnosed with facial melasma followed by CLSM analysis of their pigment distribution.[52] Furthermore, therapeutic monitoring of skin response to treatment with pyruvic acid and hydroquinone was performed in seven of these patients. The results of this study suggest that by localizing the site and extent of pigment deposits CLSM is a useful technique for the diagnosis and therapeutic follow-up for melasma.

A large cohort of 210 Asian patients was evaluated by CLSM studying the correlation of histological classification of melasma.[53] It was shown that CLSM classification of melasma corresponded well with routine histology. CLSM evaluation alone showed the predominant subtype to be epidermal ($n = 143$) followed by the mixed type yielding a total of $n = 57$ patients. These results suggest that CLSM may be a useful tool for the classification of melasma, ultimately facilitating the therapeutic management, follow-up, and prognosis on the therapeutic outcome. Pilot studies evaluating the efficacy of novel

formulations for treatment of melasma have shown promising results as to the clinical applicability of CLSM in monitoring treatment response.[54]

10 CLSM for Evaluation of UV-Induced Skin Response

Acute and repeated exposure of the skin to ultraviolet radiation results in a series of biological responses, including erythema, immediate and delayed pigment darkening, tanning, and skin color changes among the major ones. Chronic overexposure to UV radiation gives rise to genetic alterations, cell cycle arrest, and apoptosis of keratinocytes, resulting in the development of photocarcinogenesis, photoaging, and the occurence of photoallergic and photoimmunologic skin responses.

While comprehensive research has been performed on UVA-induced skin pigmentation and associated vascular changes, the specific events remain to be elucidated. Due to procedural limitations of serial histological studies, it was suggested that noninvasive imaging techniques may aid in the investigation of dynamic skin changes following UVA exposure including the evaluation of associated changes in blood flow.[39,42,55]

Yamashita et al. performed an evaluation with 27 volunteers (Fitzpatrick skin phototype SPT II-VI).[42] Following irradiation with a solar simulator, UVA-induced tanning response was evaluated by CLSM. The influence of reactive vasodilatation on immediate pigment darkening (IPD) development was examined with the addition of epinephrine this inducing vasoconstriction. Thereby, the authors have shown that CLSM detects increased capillary flow immediately after UVA radiation. One week after irradiation an increased melanization of the basal layer was observed, whereby CLSM allowed the visualization of activated melanocytes *in vivo*. The addition of epinephrine was able to partially inhibit the delayed darkening response, suggesting a role for reactive vasodilation in the consecutive events following UVA exposure.

Gambichler et al. used CLSM to assess the photoadaptive effects of repeated sunbed exposures on epidermal thickness, cell size, and epidermal pigmentation.[55] Eight volunteers were included in this study, receiving repeated sunbed exposures within a three-week period and compared to an unexposed/protected control site followed by CLSM evaluation 24 h after the last UVA-exposure. The results showed a significant increase in the thickness of the stratum corneum compared to unexposed control. There was a trend towards increased epidermal thickness, however, findings did not reach levels of significance. The size of granular keratinocytes was significantly larger in exposed versus unexposed skin and epidermal melanin content was significantly higher for UVA-exposed skin.

In that regard, a number of research studies have been aimed at the prevention of sun-induced skin changes, employing primary, secondary, and tertiary prevention strategies. Topically applied protective formulations include chemical compounds as well as physical absorbers, while systemic chemopreventive strategies have evaluated protective properties of natural antioxidants, and the administration of supplemental vitamins, plant extracts, and nutritional compounds.

For the assessment of protective properties of sunscreen formulations, the evaluation of minimal erythema dose (MED) has been established in dermatological research, allowing to grade the ability of a compound to protect against sunburns. Since MED is assessed by visual inspection, there is a high inter-, and intraobserver variability even when implementing reflectometric and chromometric methods.[56–58]

Gambichler et al. have performed a correlative study evaluating the dynamic skin changes following exposure to a solar simulator at $1 \times$ MED and $3 \times$ MED using CLSM in a total of 10 volunteers.[59] The findings of this study showed an increase in epidermal thickness, the presence of spongiosis and an increased pigmentation of the basal layer in a dose/time-dependent manner. In addition, CLSM allowed the visualization of reactive vasodilatation of dermal capillaries.

Ulrich et al. performed an evaluation on cutaneous changes following UVB-irradiation, including the evaluation a commercially available sunscreen compared to untreated control.[60] A total of five patients Fitzpatrick SPT II and III were enrolled in this pilot study, followed by irradiation of the volar forearm using a commercially available UVB light source with five gradually increasing dosages, whereby test sites with sunscreens were compared to contralateral controls receiving no sunscreen. Clinical and CLSM evaluations were performed at four consecutive time points.

The findings of this study showed that CLSM permits the visualization of inflammatory response *in vivo*, including the visualization of single apoptotic cells (sunburn cells) as well as

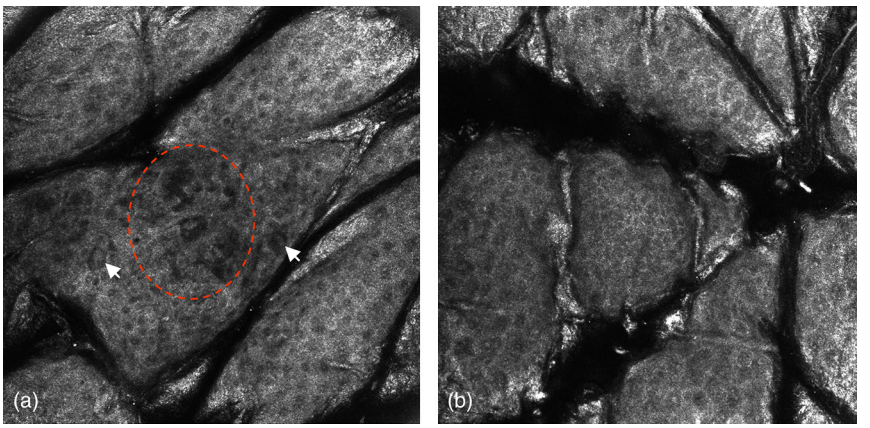

Fig. 4 Representative RCM images of the granular/spinous layer 24 h after irradiation with UV-B (240 J/cm^2) (a) without sunscreen and (b) after use of sunscreen. (a) The RCM image (500×500 μm) illustrates the presence of cells with bright center and dark periphery, corresponding to sunburn cells (white arrow). Furthermore, small microvesicles can be noted (red dashed circle). Part (b) was obtained at the contralateral arm of the same patient after sunscreen application. The RCM image (500×500 μm) shows regular honeycomb pattern of the epidermis without any signs of sun damage.

microvesicle formation. The presence of inflammatory changes and sunburn cells correlated with respective UVB doses and the respective susceptibility (skin phototype) Lastly, it was shown that following the topical application of sunscreen formulations at standard dosage no UVB-induced skin changes were detected using CLSM (Fig. 4).

11 Conclusions and Future Perspectives

Reflectance confocal microscopy represents a promising technique which has shown its usefulness for a wide range of scientific as well as clinical applications. In the past years, *in vivo* CLSM has developed from a research tool to a valuable adjunct diagnostic tool providing the opportunity for noninvasive evaluation of skin lesions with histological detail. Thereby, CLSM has become an important part in the evaluation of skin cancer in academic clinical settings as well as specialized outpatient and research centers. Furthermore it allows the noninvasive monitoring of topical therapies as well as an assessment of efficacy for cosmetic products and laser procedures.

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