Lasers Med Sci (2012) 27:261–266
DOI 10.1007/s10103-011-0943-9

BRIEF REPORT

Noninvasive visualization of intraepidermal and subepidermal blisters in vesiculobullous skin disorders by in vivo reflectance confocal microscopy

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Received: 21 April 2011 / Accepted: 1 June 2011 / Published online: 6 July 2011

Introduction

Bullous dermatoses are characterized by skin blistering resulting from local injury with breakdown of tissue integrity and fluid accumulation within specific layers of the skin. These disorders are traditionally classified into subcorneal, suprabasal, and subepidermal blistering by the specific location of the split in the epidermis [1, 2]. Of special interest is the group of autoimmune blistering diseases that comprise a wide spectrum of clinical presentations and are mediated by pathogenic antibodies targeting specific adhesion molecules responsible for cutaneous homeostasis and integrity [3]. Diagnosis is based on the clinical picture, histology, direct and indirect immunofluorescence, immunoblotting, immunoprecipitation and immunoelectron microscopy [4, 5]. Though crucial for accurate diagnosis and for selection of specific therapy, these techniques are cumbersome, time-consuming and unlikely to be widely available, leaving blister level determination by classical histology a key diagnostic procedure in intraepidermal and subepidermal blistering diseases.

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Reflectance confocal microscopy (RCM) is a novel, noninvasive imaging technique which permits real time visualization of cellular components in the skin at a resolution compatible with that of conventional histology [6]. RCM detects single back-scattered photons directly from illuminated living tissue without prior preparation of the examined skin. A small pinhole in the front of the detector allows imaging at high resolution. Contrast in confocal images is provided by the differences in refractive index among the cellular organelles and structures. Melanin acts as a contrast agent in pigmented epithelia [7]. With the current technology, in vivo RCM imaging is limited to a depth of approximately 300 μm which includes the entire epidermis, the papillary dermis and the upper reticular dermis. It is of interest that the depth of RCM imaging can be increased by using the so-called "optical clearing" approach, and experimental studies utilizing gold nanoparticles and osmotically active immersion liquids as optical clearance agents have indeed increased the imaging depth of RCM up to three times [8–11]. However, these experimental approaches have not yet been incorporated into clinically useful RCM technology. Since contrast in the images is primarily provided by melanin, RCM has mainly been useful in the diagnosis of pigmented cutaneous tumors such as melanoma and nevi [12], and nonmelanoma skin cancers [13, 14]. The use of RCM has also been reported in the diagnosis of a variety of inflammatory skin disorders including psoriasis [15], contact dermatitis [16, 17], vitiligo [18], cutaneous lupus erythematosus [19, 20], folliculitis [21], and photoaging [22]. In a recent paper, Angelova-Fischer et al. [23] demonstrated the use of RCM in the diagnosis of subcorneal blisters in two patients with pemphigus foliaceus in which the dark nonrefractive blister cavity was readily visible against a background of bright,

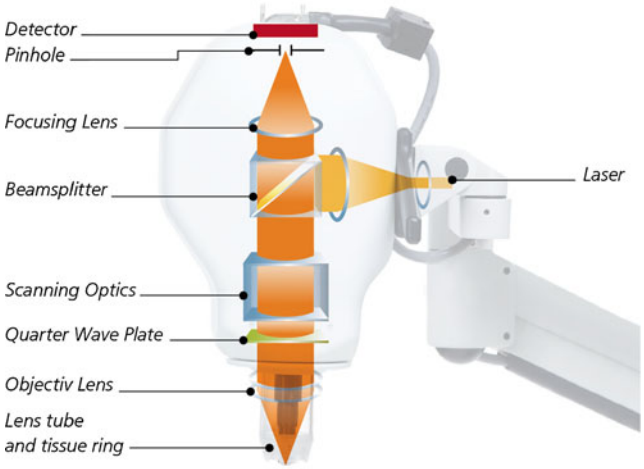

Fig. 1 Confocal microscope

highly reflective cells of the stratum corneum reaching down to the stratum granulosum.

Based on the ability of RCM to scan the entire depth of the epidermal compartment, we hypothesized that RCM could also be useful in noninvasive determination of the blister level in vesiculobullous skin disorders characterized by blister formation in the deeper layers of the epidermis and dermis. We here demonstrate how a "virtual" biopsy by RCM accurately differentiates between subcorneal, suprabasal, and subepidermal blistering in pemphigus foliaceus, pemphigus vulgaris and bullous pemphigoid.

Methods

In vivo RCM was performed using a commercially available near-infrared reflectance confocal microscope (Vivascope 1500; Lucid, Rochester, NY). The system uses an 830-nm wavelength diode laser. The laser beam is directed by a dichromatic mirror (beam splitter) towards a pair of mirrors (scanning optics) that scan the selected skin area horizontally. The laser beam then passes through the microscope objective lens and excites fluorescence within the skin. Next, the emitted light goes back through the objective lens and the dichromatic mirror which focuses it onto a pinhole. The light that passes through the pinhole is measured by a photomultiplier detector (Fig. 1).

The system provides high optical resolution (horizontal axis 2.0 μm; vertical axis 5.0 μm) to a penetration depth of 200–300 μm dependent on anatomical site and skin thickness.

An ultrasound gel was used as immersion medium (refractive index 1.33). Images (500×500 μm) of en face

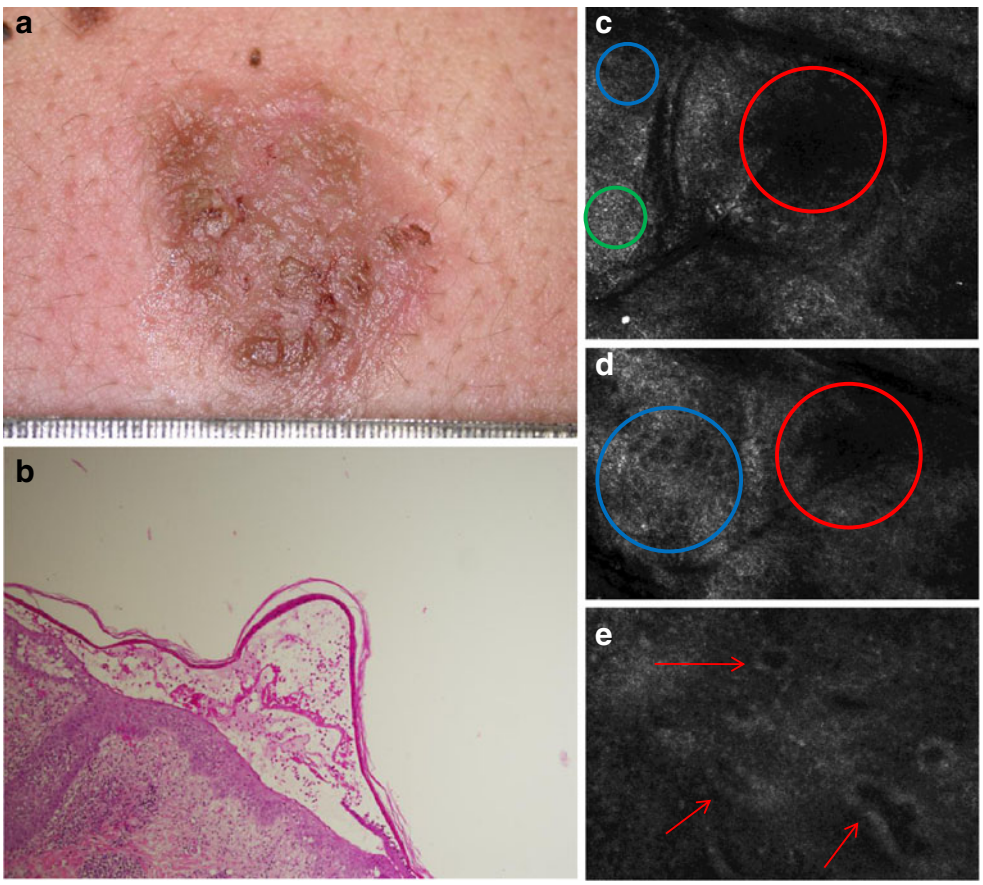

Fig. 2 Pemphigus foliaceus. **a** An erosion on the upper back. **b** Histology shows an intraepidermal split with acute inflammation and acantholytic cells. RCM images: **c** a hyporefractive blister cavity (*red circle*) is present at the level of the stratum corneum (*green circle* characteristic hyperrefractive area in the stratum corneum, *blue circle* characteristic honeycomb pattern). **d** a blister (*red circle*) extends through the stratum granulosum (*blue circle* characteristic honeycomb pattern). **e** the blister is closed at the level of the dermal epidermal junction (*arrows* dermal papillae)

sections of the skin from the stratum corneum to the upper dermis were taken at intervals of 3.0 μm. At least five images, corresponding to the stratum corneum, the stratum granulosum, the stratum spinosum, the stratum basale/dermoepidermal junction and the upper dermis were obtained and stored in BMP file format as described previously [23]. The blister cavity was readily detectable as a dark homogeneous nonreflective space, and the blister level was determined by examining the morphological features of the cells surrounding the blister cavity.

Results

Pemphigus foliaceus

The clinical, histological and RCM imaging findings as seen in two patients with pemphigus foliaceus are shown in Fig. 2. Superficial erosions were seen clinically (Fig. 2a) and histological examination showed epidermal detachment accompanied by secondary acute inflammation with fibrin deposition and acantholytic cells within the blister cavity (Fig. 2b). Direct immunofluorescence showed IgG antibodies in the intercellular space. RCM showed a subcorneal blister extending from the superficial epidermis and closing at the level of the stratum spinosum/dermoepidermal junction. (Fig. 2c). The RCM findings were consistent in both patients.

Pemphigus vulgaris

The clinical, histological and RCM imaging findings as seen in three patients with pemphigus vulgaris are shown in Fig. 3. Widespread scattered flaccid blisters and oral mucosal erosions were seen clinically (Fig. 3a) and histological examination showed suprabasal blistering with

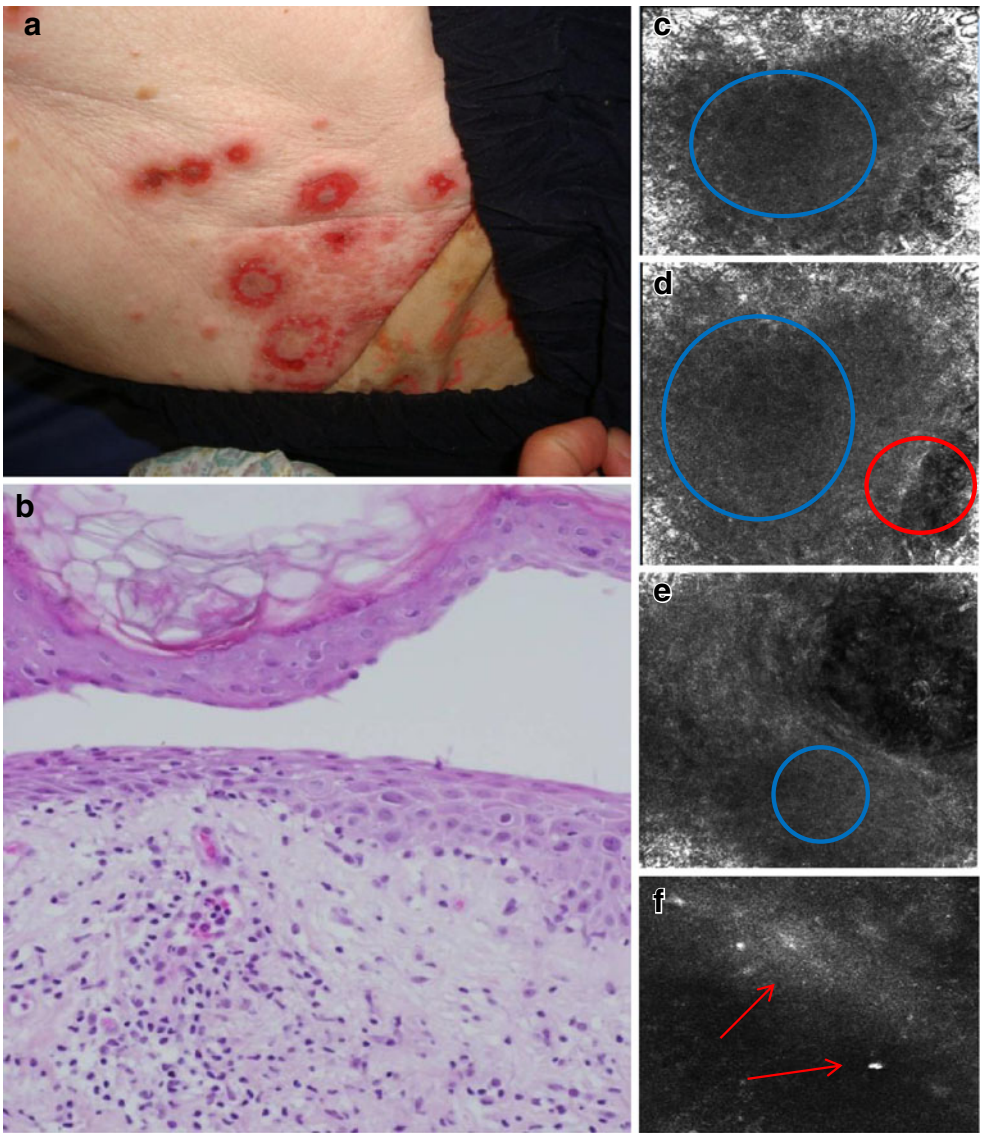

Fig. 3 Pemphigus vulgaris. **a** Flaccid blisters on the back. **b** Histology shows suprabasal separation. RCM images: **c** intact stratum granulosum (*blue circle* characteristic honeycomb pattern). **d** initial blister formation (*red circle*) at the level of the stratum granulosum/spinosum (*blue circle* characteristic honeycomb pattern). **e** blister extension throughout the stratum spinosum (*blue circle* characteristic honeycomb pattern). **f** the blister is closed at the level of the upper dermis (*arrows* dermal capillaries)

acantholysis (Fig. 3b). Direct immunofluorescence showed IgG antibodies in the intercellular space. Indirect immunofluorescence showed IgG antibodies at titers of 1/20 to 1/40. RCM showed a blister cavity at the level of the stratum granulosum/spinosum extending throughout the epidermis and reaching the upper dermis (Fig. 3c). The RCM findings were consistent in all three patients.

Bullous pemphigoid

The clinical, histological and RCM imaging findings as seen in three patients with bullous pemphigoid are shown in Fig. 4. Multiple, tense, clear blisters occurring on an urticarial background were seen clinically (Fig. 4a). Skin biopsy showed a subepidermal blister filled with eosinophilic infiltration and fibrin; mononuclear and eosinophilic infiltration was noted throughout the dermis (Fig. 4b). Direct immunofluorescence showed IgG antibodies in all three patients and C3 in two patients at the dermoepidermal junction. RCM showed a blister at the level of the dermoepidermal junction reaching down to the upper dermis (Fig. 4c). The RCM findings were consistent in all three patients.

Discussion

The intraepidermal and subepidermal blistering diseases are characterized by loss of epidermal adhesion (acantholysis) and the presence of pathogenic IgG antibodies targeting various cell adhesion molecules. The specific location in the epidermis of these adhesion molecules determines the level of the intraepidermal split. In spite of the fact that our understanding of the molecular structure of the epidermis and the pathogenesis of the autoimmune blistering diseases has increased dramatically in recent years [24], histological characterization of the level of the intraepidermal split remains a crucial determinant in the diagnosis of these diseases. RCM of lesions from a series of eight patients with pemphigus foliaceus, pemphigus vulgaris, and bullous pemphigoid were examined and showed hyporefractive blister formation at the level of stratum granulosum, stratum granulosum/spinosum, and the dermoepidermal junction, respectively—findings that closely correlate with conventional histology in these disorders.

RCM provides a "virtual biopsy" of the skin lesion covering the various epidermal layers down to approximately 300 μm, thus spanning the whole epidermis, the papillary

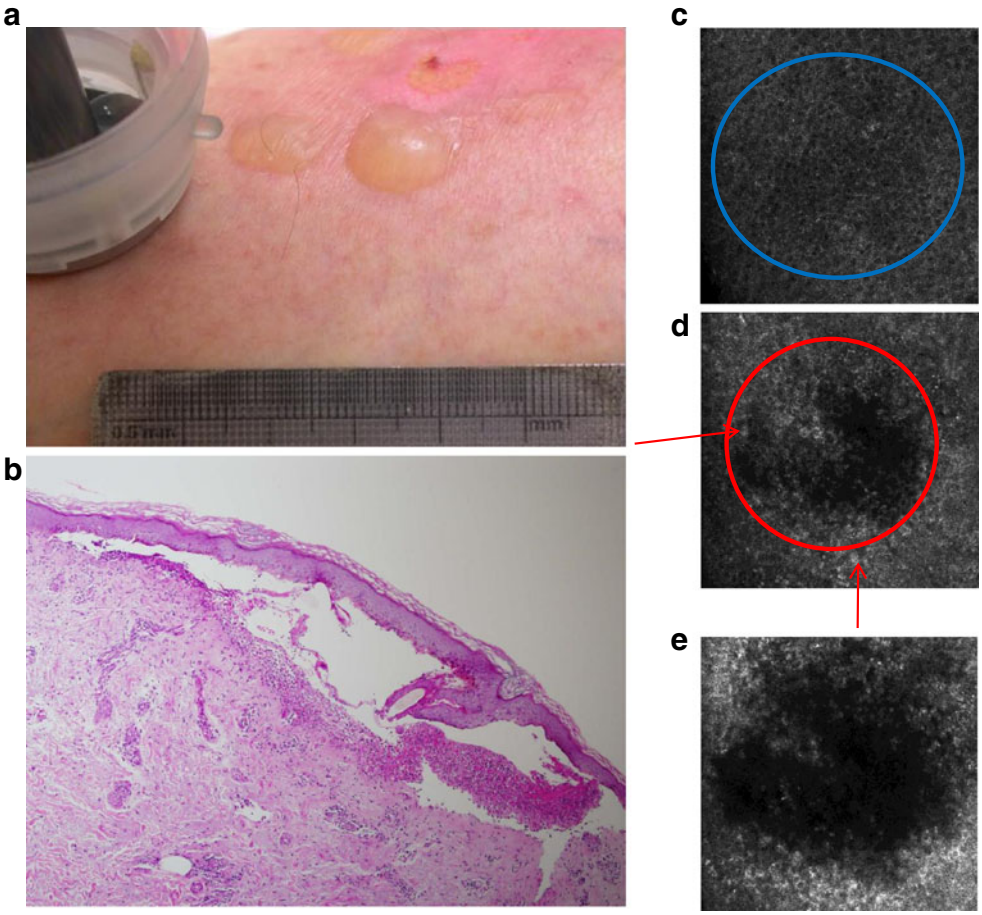

Fig. 4 Bullous pemphigoid. **a** Tense blisters (confocal microscope probe is seen at the left upper corner). **b** Histology shows subepidermal blister. RCM images: **c** intact stratum spinosum (*blue circle* characteristic honeycomb pattern). **d** initial blister formation (*red circle*) at the level of the dermoepidermal junction (*arrows* basal cells with a hyperrefractive center. **e** blister extension throughout the upper dermis

dermis and the superficial parts of the reticular dermis. Since the blister fluid has low refractance compared to the surrounding cellular components, the space taken up by the blister will appear dark compared to the brighter surroundings. Since RCM provides visualization of the skin structures in a horizontal plane, serial horizontal "cuts" at various depths are required to determine the full extension of the blister space within the epidermis.

RCM is a novel imaging tool developed primarily for noninvasive diagnosis of pigmented lesions [6], but its use is increasingly being reported in various inflammatory skin disorders [15–23]. In the present report we have expanded the list of skin disorders in which RCM can be useful also to include the whole range of bullous diseases spanning from subcorneal via suprabasal, down to subepidermal blisters represented by pemphigus foliaceus, pemphigus vulgaris and bullous pemphigoid, respectively. Further studies of blistering disorders might expand this list to also include impetigo (subcorneal), erythema multiforme and staphylococcal scalded skin syndrome (intraepidermal), benign familial pemphigus, transient acantholytic dermatosis and epidermolysis bullosa simplex (suprabasal), and dermatitis herpetiformis, bullous lichen planus, urticaria pigmentosa, porphyria cutanea tarda, acute graft-versus-host reaction and toxic epidermal necrolysis (subepidermal).

In contrast to the application of RCM to pigmented lesions where diagnosis is often based on complicated algorithms weighting the presence of a long list of cellular and structural pathologies, the application of RCM to the determination of the blister level appears especially attractive since the blister space in readily identifiable and the split level can be determined directly by identifying the surrounding epidermal cells. Thus, minimal training in RCM technology is required to obtain a quick and reliable determination of the blister level. Future improved RCM image technology [8–11] may result in even easier and more accurate blister level determination.

Furthermore, conventional biopsying of blistering diseases is notoriously hampered by the difficulty in obtaining representative samples of the blister tissue because of the superficial location of the blisters that easily rupture following even minimal manipulation during the biopsy procedure. In contrast, in vivo RCM technology allows direct visualization of the blister tissue in situ, and the noninvasive nature of the procedure as opposed to conventional biopsies easily allows sampling of multiple lesions in the same patient, thereby increasing the probability of obtaining representative samples. Though no direct comparison with classical histology in terms of sensitivity and specificity was attempted in the present study, the possibility of noninvasive multiple sampling by RCM suggests that increased validity (sensitivity and specificity) is attainable using RCM technology.

In summary, we have demonstrated the usefulness of in vivo RCM in determining the blister level in pemphigus foliaceus, pemphigus vulgaris and bullous pemphigoid—three vesiculobullous skin diseases representing subcorneal, suprabasal and subepidermal blistering, respectively. However, although determination of the blister level is directly helpful in the work-up of such patients, it should be noted that a final diagnosis rests on additional immunological and molecular characteristics which at present cannot be addressed by RCM technology.

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