

ORIGINAL ARTICLE

Fluorescent fibre-optic confocal characterization of in vivo epidermal changes in atopic eczema

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Abstract

Background/aims: Fibre-optic confocal imaging (FOCI) allows non-invasive visualization of live skin in vivo. A contrast agent, a fluorophore, is injected into the dermis. FOCI images are horizontal optical sections with cellular resolution. The aim was to study in vivo epidermal changes and the cellular structure of keratinocytes in moderate to severe atopic eczema (AE).

Methods: Eight patients with AE with active lesions on the forearms were studied and compared to a control group of six healthy individuals, and two cases of AE without activity. Fluorescein sodium was used as fluorophore. A hand-held fibre-optic laser scanner (Stratum[®]) was used. The study included morphometric analyses.

Results: The confocal in vivo images identified characteristic features of epidermis and keratinocytes in active AE vs healthy skin controls. FOCI could non-invasively image *acanthosis*, *spongiosis*, and *parakeratosis* in AE. Epidermal oedema and micro-vesicles were visualized. Morphometry based on FOCI demonstrated 14% increased width of keratinocytes of atopic skin vs healthy controls. The epidermal structures and organization in distinctive cell layers were deviant as a result of the disease.

Conclusions: Fibre-optic confocal imaging can visualize essential epidermal structures of atopic eczema directly in vivo, in real-time, and with cellular resolution thus without disturbing the natural state of the skin. FOCI is primarily a research tool, but with a potential to become used in the clinic for non-invasive microscopic diagnosis of AE and monitoring of effect of therapies.

KEYWORDS

acanthosis, atopic eczema, confocal imaging, keratinocyte, morphometry, parakeratosis, spongiosis

1 | BACKGROUND

Various non-invasive tools for evaluation of the skin in its natural state were recently introduced. Reflectance confocal laser microscopy (RCM) in vivo can be used in studies of epidermal cells in various inflammatory skin diseases.¹⁻⁵ Atopic skin has been studied by RCM.⁵

We have applied another mode of confocal microscopy namely fluorescent fibre-optic confocal imaging (FOCI), enabling

visualization of the epidermis and outer dermis at higher cellular resolution, for example, at 1000× magnification.⁶

Atopic eczema (AE) is a common skin disease in children dominated by eczema, inflammation, and itch.⁷ Adults also may suffer from AD. In recent years, the focus has been on epidermal barrier dysfunction measured by increased trans-epidermal water loss (TEWL).⁸ The pathology includes increased desquamation, loss of corneocyte cohesiveness, and extracellular lipid contents, thus, changes which might be imaged by FOCI.

The disease has various stages depending on the degree of inflammation in the skin. In histopathology, dilated blood vessels and inflammatory cells migrated into the epidermis resulting in both intracellular and extracellular oedema in stratum spongiosum are described under the term *spongiosis*. Mechanical shearing of inter-cellular attachment sites, for example, desmosome anchoring, and disturbance of cell membranes by progressive accumulation of inter-cellular fluid may result in the formation of intraepidermal vesicles.⁹ The epithelium becomes thickened manifested as *acanthosis* with excessive keratin production in the clinic manifested as hyperkeratosis and scaling. The skin is red, dry, scaly, thickened, and with a tendency to cracking and fissuring.¹⁰ The diagnosis of AE is primarily based on clinical observation; there is no specific histology or biochemical marker.

Previous *in vivo* studies using RCM suggest that there are distinct objective features when analysing atopic and healthy skin at the cellular level.⁵ It is hypothesized that keratinocytes can change in size and structure in afflicted skin. In this article, we will report our observations of the epidermis in adults with atopic eczema compared to healthy skin controls. Morphometry and statistical analysis will be applied.

2 | MATERIALS AND METHODS

2.1 | Participants

Ten patients aged 39–51 years (mean 44 years, seven women and three men), with a diagnosis of atopic eczema, all with debut in early childhood, were recruited by poster invitation in waiting room of our clinic. The volunteers received written information about the study. Exclusion criteria were any topical treatment on the day of examination, UVA/UVB treatments in the week preceding inflammation, ongoing systemic treatments, immunodeficiency, pregnancy, age under 18 years, severely excoriated and fissured skin following scratching, and secondary bacterial infection. The controls were aged 36–48 years (mean 43 years, four women and two men) were healthy individuals having no skin disorders. Furthermore, two patients with a past history of atopic dermatitis, however, presently without active dermatitis were studied as a reference to active disease. Ethics approval was granted by the regional Ethics Committee. Informed written consent was obtained before examination.

2.2 | Clinical grading of severity of atopic eczema

The EASI scoring system (Eczema Area and Severity Index) was chosen for grading of the clinical severity of active atopic dermatitis.^{11–16} Erythema, oedema/papulation, excoriation, and lichenification were examined and assessed on the subjects using an examination lamp. Intensity criteria were ranged from 0–3 (0: none, 1: light, 2: moderate, 3: severe). All parameters contributed to the final EASI scores measuring activity of the entire skin. The upper extremities were graded as a separate anatomical region.

2.3 | Study sites, applications and injection of fluorophore

The right forearm was chosen for FOCI examination performed in three anatomical sites: the cubital fossa (CF), the flexor side middle forearm (MF), and the flexor side of the wrist (WR). The study sites were disinfected by wiping with a standard alcoholic preparation (Softasept™ N, B. Braun). A droplet of fluorescein sodium (Alcon Pharma GmbH) diluted to 0.1% w/v concentration in 0.9 g/L saline solution was applied topically and spread uniformly to the sites.

A volume of 20 µL of the fluorescein sodium was injected in the dermis immediately under the epidermis at the three selected test sites, using a fine needle. Scanning started after 5 minutes. Standard immersion microscopy oil (Carl Zeiss, refractive index $n = 1.518$) was used as coupling medium between the scanner probe and the skin surface. The same procedure and the same anatomical sites were used in the controls.

2.4 | Confocal laser scanning microscopy

Fibre-optic confocal imaging was accomplished with the Stratum manufactured by Optiscan Pty Ltd.⁶ The intensity of the fluorescent signal was digitized to a 12-bit grey scale for measurement, and images were scanned at 1024 × 1024 pixels/image, captured at 1.4 s/frame. The resulting images had an optical resolution of <1 µm laterally and <3 µm axially. The scanned area was 200 × 200 µm. Images were saved in bitmap format.

2.5 | Visual assessment of images

The assessment of the FOCI images was performed by subjective visual inspection by the Authors, concluded according to the known

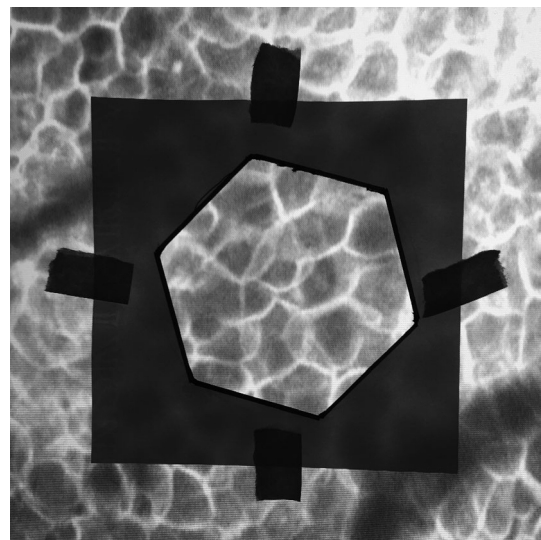


FIGURE 1 Hexagonal template used for cell counting in a fixed area

TABLE 1 Morphometric data and observations in subjects 1-8 with active atopic dermatitis (A) and subjects 9-10 with a history of previous atopic dermatitis and in the healthy controls numbered 11-16 (B)

Subject No.	Gender	Age (years)	Total EASI Score	EASI Score upper extr	Max cell size (µm)				Cells/hexagon (n)				Mean	Parakeratosis	Furrows	Acanthosis	Spongiosis
					CF	MF	WR	Mean	CF	MF	WR	Mean					
(A)																	
1	F	40	5.6	16	22	19	20	20.3	42	45	47	44.7	Yes	Yes	Yes	Yes	Yes
2	F	45	18.4	5.2	14	20	22	18.7	78	50	48	58.7	No	Yes	Yes	Yes	Yes
3	F	48	5.5	1.6	20	17	23	20.0	43	49	40	44.0	No	Yes	Yes	Yes	Yes
4	F	40	2.5	0.8	20	21	21	20.7	62	56	63	60.3	No	No	Yes	Yes	No
5	M	39	21.4	3.0	21	15	17	17.7	46	54	50	50.0	No	Yes	Yes	Yes	Yes
6	M	49	4.8	1.2	22	19	19	20.0	32	74	63	56.3	No	No	Yes	Yes	Yes
7	F	45	21.5	4.0	22	24	21	22.3	47	46	46	46.3	Yes	Yes	Yes	Yes	Yes
8	F	48	2.0	1.2	18	19	18	18.3	52	52	46	50.0	No	No	No	No	No
Mean	-	-	10.2	2.3	20	19	20	19.8	50	53	50	51.3	-	-	-	-	-
SD	-	-	8.6	1.6	2.7	2.7	2.0	1.5	14	9.2	8.3	6.4	-	-	-	-	-
(B)																	
9	F	49	0	0	17	14	16	15.7	66	80	72	72.7	No	No	No	No	No
10	M	51	0	0	17	18	17	17.3	76	74	66	72.0	No	No	No	No	No
11	F	36	0	0		18				52							
12	F	48	0	0		15				69							
13	M	39	0	0		19				45							
14	F	40	0	0		16				67							
15	F	46	0	0		19				46							
16	M	47	0	0		17				49							
Mean	-	43	-	-	-	17.3	-	-	-	55	-	-	-	-	-	-	-
SD	-	5	-	-	-	1.6	-	-	-	10.6	-	-	-	-	-	-	-

Note: Mean and SD in B refers to subjects 11-16.
Abbreviations: CF, cubital fossa; MF, middle flexor forearm; WR, flexor side wrist.

standard for epidermal pathology evaluation practised in histopathology. *Acanthosis* was defined as thickening of the dermo-epidermal interface with elongation of rete ridges, *spongiosis* as intracellular oedema with widening of the spaces between keratinocytes, and *parakeratosis* as visible cell nuclei reaching the stratum corneum. FOCI images from diseased skin were referenced to images obtained in healthy skin.

2.6 | Morphometry

The number of cells in a fixed area was determined by using a *hexagonal template* corresponding to 20% of the total field of view, Figure 1. This was a practical method as keratinocytes are honeycombed in vivo when observed in the horizontal plane; that is, they have spontaneously a hexagonal form. Cell counting was done in or nearby the central area of the scanned image. *Cell density* defined as the number of well-defined cells per hexagon was determined. *Cell size* of selected keratinocytes in the horizontal plane was measured. For the purpose of standardization and precision, the largest keratinocyte in an image was identified and the largest diameter measured, expressed in micrometres. Small- and medium-sized keratinocytes were variably displayed and were not adequate objects for measurement. Images were analysed and assessed directly from the screen or from hardcopies. Cell counting and size measurement were also done by an assistant in order to secure unbiased data. When there were discrepancies, an average value was chosen.

2.7 | Statistical calculations

The statistical analysis was done using a model assuming normal double-side distribution. Student's *t* test with weighed variances was used for hypothesis testing. It was hypothesized that there were no differences between study group and controls. This was done at different test levels. Spearman's rank order correlation coefficient test was also applied.

3 | RESULTS

Study results obtained with FOCI are shown in Table 1A,B. The table includes observations in the groups of active atopic eczema (probands 1-8, *n* = 8), in atopy without active disease (probands 9-10, *n* = 2), and in healthy controls (probands 11-16, *n* = 6).

The control group and the group without active disease were analysed with by regression, maximum cell size vs number of cells/hexagon; *r* was $-.81$, see Figure 2. Box plot diagrams of the study results are shown in Figures 3 and 4.

3.1 | Visual assessments of FOCI images

Reference images of healthy skin are shown in Figure 5A,B. In the images of atopic skin, *acanthosis*, *spongiosis*, *parakeratosis*, and

abnormal furrows indicating lichenification were observed, see Table 1A and Figure 6. Enlarged keratinocytes and overlapping of cells in stratum spinosum are illustrated in Figures 5 and 7-11. The figures also illustrate in Figure 9A,B the finding of cell nuclei in the outermost epidermis as sign of *parakeratosis*. Epidermal inter-cellular oedema occasionally with micro-vesicles is also visualized. Image scale is 200×200 microns except in Figure 1.

3.2 | Morphometry of FOCI image

Morphometric data were collected from the epidermis corresponding to the basal layer, as shown in Table 1A,B.

- Statistical calculation showed (*t* test and Spearman) as follows:

No correlation between cell size of selected keratinocytes of large extension and cell density, that is, number of cells in the hexagon frame, by the Spearman correlation coefficient.

- It is notable that the maximum overall cell size in lesions in the main study group (probands 1-8) ranged from 14 to 24 μm compared to 15 to 19 μm in healthy controls. The group with inactive AE (probands 9-10) ranged from 14 to 18 μm . See Figure 3.
- Maximum cell size in patients with active AE in the cubital fossa and flexor side wrist against middle flexor forearm showed no significant differences ($P = .05$).
- Maximum cell size in the cubital fossa was increased compared to controls ($P < .05$). The mean increase was 16%.
- Maximum cell size in diseased skin in the flexor wrist vs controls was increased ($P < .01$). It ranged from 17 to 23 μm compared to 15 to 19 μm in controls. The mean increase was 16%.
- Number of cells in the cubital fossa and flexor side wrist against flexor side forearm were not different ($P = .05$).

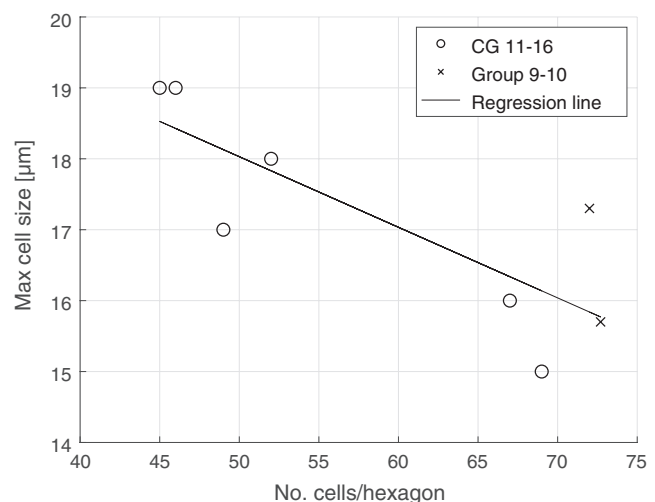


FIGURE 2 Max cell size in μm vs number of cells/hexagon, $r = -.81$, in healthy control subjects 11-16 (circular point) and subjects 9-10 (cross) with previous atopy

- Number of cells/hexagon in lesions compared to number of cells/hexagon in controls showed no significant difference ($P = .05$).
- The mean maximum cell size was increased in diseased skin vs controls ($P < .01$). It ranged from 17.7 to 22.3 μm compared to 15 to 19 μm in controls. The mean overall cell size was increased by 14% in lesions, compared to controls.
- Mean number of cells/hexagon in lesions compared to number of cells/hexagon in controls showed no difference.
- The correlation coefficient of maximum cell size vs number of cells/hexagon in controls was $r = -.93$, thus, a highly significant negative correlation ($P < .01$). Larger cell size also correlated to lower cell area density in the group of healthy controls. The study

group inactive AE showed $r = -.81$. Correlations are illustrated in Figure 2.

- Maximum overall cell size vs mean number of cells/hexagon in the main group of AE showed no difference.
- There was no correlation of importance in the main study group with lesions when analysing total EASI score vs mean maximum cell size. There were several outliers. When analysing EASI score for upper extremities vs mean maximum cell size there was no correlation of importance.
- Analysing total and upper extremities EASI score vs mean number of cells/hexagon no correlation was found.
- EASI scores vs mean maximum cell size in lesions showed no correlation.

FIGURE 3 Maximum cell size in μm in subjects 1-8 with active atopy, 9-10 with previous atopy, and in healthy controls numbered 11-16. Mean and SD of subjects 1-8 is shown. Three anatomical sites were studied

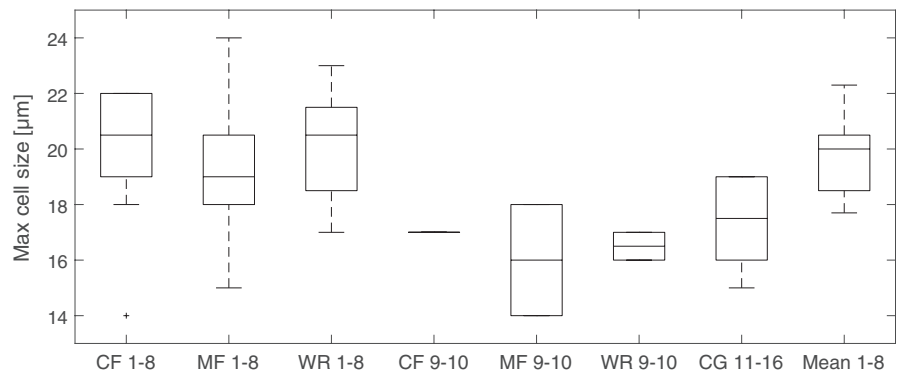


FIGURE 4 Number of cells/hexagon (n) - in subjects 1-8 with active atopy, 9-10 with previous atopy, and in healthy controls numbered 11-16. Mean and SD of subjects 1-8 is shown. Three anatomical sites were studied

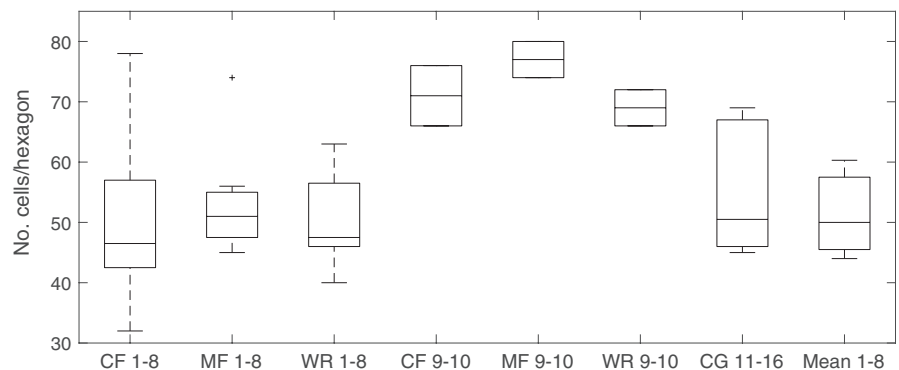
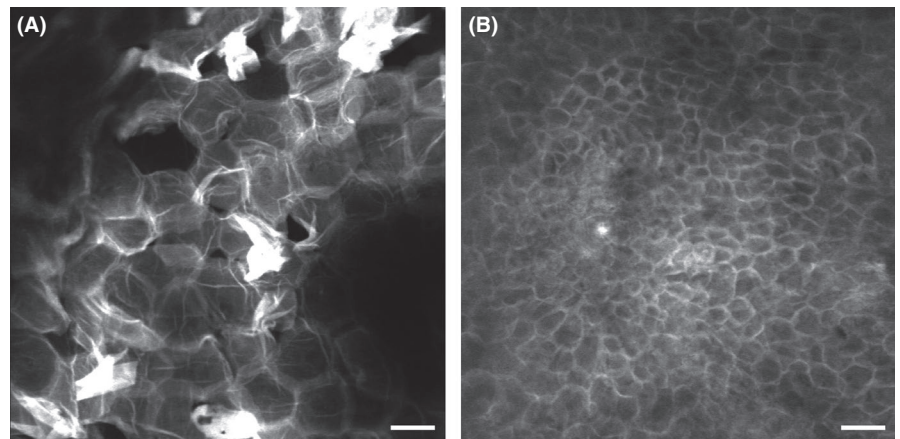


FIGURE 5 A, Reference image of stratum corneum of healthy skin. Scale bar: 20 μm . B, Reference image of the Malpighian layer of healthy skin. Scale bar: 20 μm



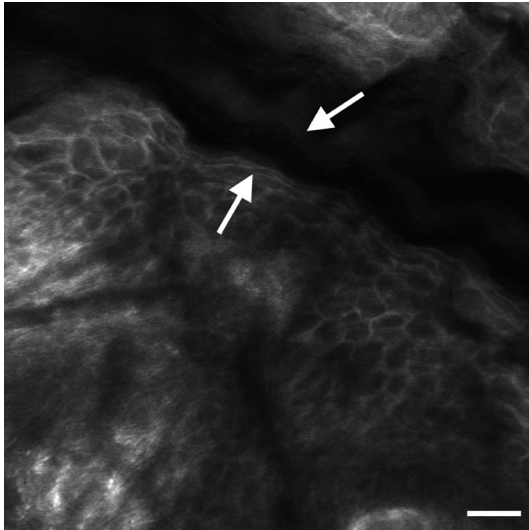


FIGURE 6 Deep epidermal furrows. Scale bar: 20 μ m

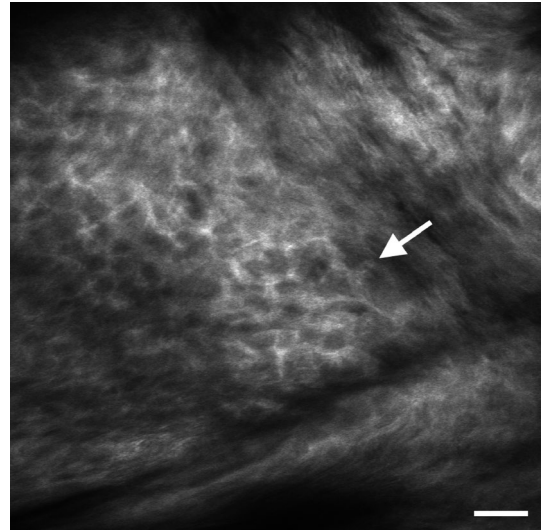


FIGURE 8 Oedema of stratum spinosum (spongiosis). Scale bar: 20 μ m

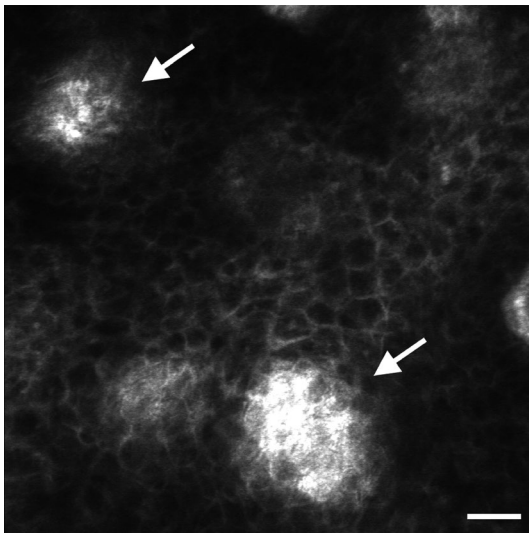


FIGURE 7 Thickening of stratum spinosum with marked dermal papillae (acanthosis). Scale bar: 20 μ m

4 | DISCUSSION

The study showed that FOCI is a method of optical biopsy that can depict subclinical features known from histology such as *acanthosis*, *spongiosis*, and *parakeratosis*. Demonstration of *parakeratosis* by FOCI is to the best of our knowledge a new and original observation. These markers of severity and activity of eczema known since long time may guide the clinician in the decision on therapy, which can be local or systemic. The limitation of FOCI in the perspective of clinical practice is the cost of the equipment, the need for expertise and time used to obtain and analyse images. The method and the application to atopic dermatitis are mainly directed towards research.

Morphometry by FOCI in active atopic eczema referenced to healthy skin demonstrated increased keratinocyte width of 14%.

The lacking correlation between cell size and cell density in atopic eczema, contrasting a significant correlation in healthy skin, indicates increased variability of cells of diseased epidermis regarding their structure and size as also might be expected, manifested in pathological patterns of *acanthosis*, *parakeratosis*, and *spongiosis*. These classical patterns from histopathology can be identified by FOCI, with no cut and no surgical sample.

Being a high magnification method, FOCI shares the limitation of comparative methods for high-resolution study that only a very small fragment of the diseased skin can be evaluated, with the risk that findings can be very different in samples obtained from neighbouring sites of the same main lesion. Thus, exactly where the FOCI scan is made may be a critical prerequisite. Findings can become more valid if scanning from different local sites is reproducible. With the FOCI in contrast to punch biopsy and histopathology, a number of local sites are easily examined.

Cell counting per hexagon in the diseased study group was complex due to overlapping of cells and structural variability. Counting could not always be done at the preferred position in the FOCI image. Ideally, counting cells in the centre of the image is preferable considering the operation of the scanner. Mechanical shearing of inter-cellular attachment sites including desmosomes and cell membranes may occur due to the increase in cell size, causing cellular overlap and thus blurring cell borders, complicating cell counting in a fixed position. The undulating nature of live epidermis with movement artefacts is also a relevant confounder limiting the method.

Topical application of fluorescein sodium allows a relatively short imaging time lasting only a few minutes where after the fluorophore is washed out. Also, intradermal injection and the local spreading of the fluorophore in the tissue are variable. This problem has been discussed in previous studies.¹⁷ Researchers have introduced a lotion containing fluorescein sodium, which produces considerably longer imaging time, lasting for hours.¹⁸ This method could be used in future studies of AE.

FIGURE 9 A, Retained cell nuclei in stratum corneum (parakeratosis). Scale bar: 20 μ m. B, Retained cell nuclei in stratum corneum (parakeratosis). Scale bar: 20 μ m

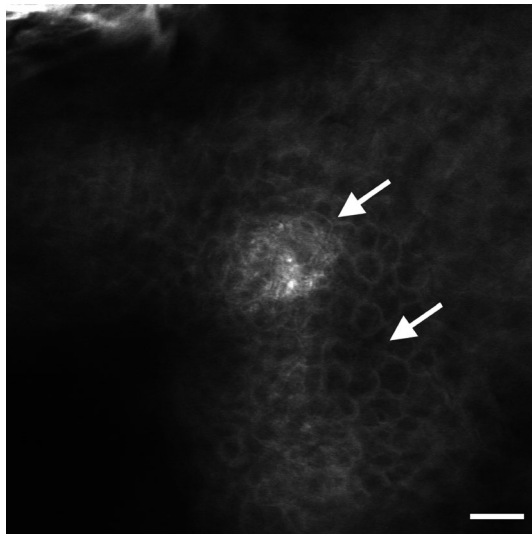
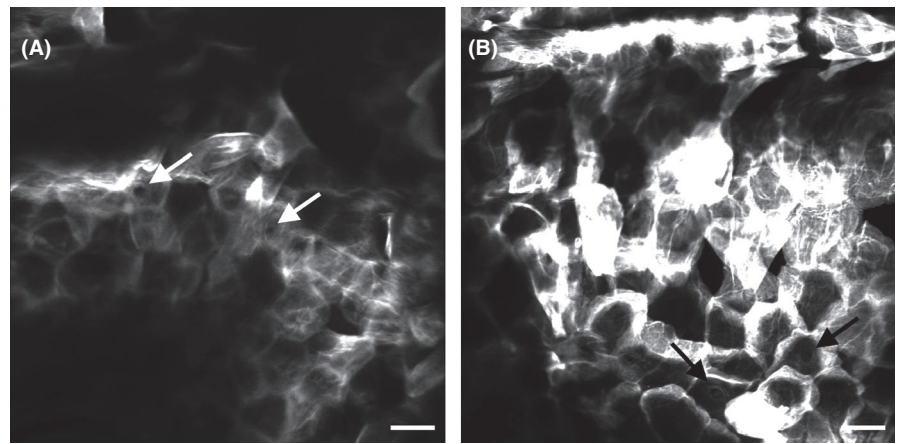


FIGURE 10 Overlapping of cells. Dermal papilla is seen in the centre of the figure. Scale bar: 20 μ m

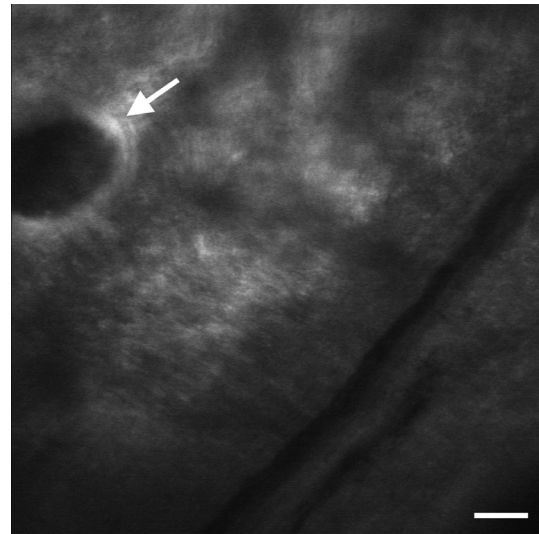


FIGURE 11 Micro-vesicles and epidermal oedema. Scale bar: 20 μ m

Multiphoton laser tomography, another new high-resolution imaging method, offers imaging of cells and even visualization of intracellular organelles. However, the time for image capturing such image can be up to 17 minutes.¹⁹ The multiphoton system is expensive and requires high technical expertise to run; it is for centres of excellence.

Optical biopsy by FOCI is presently mainly a research tool. However, the method has additional to experimental research a potential for objective diagnosis and disease monitoring in the clinic due to non-invasiveness and high resolution of the method.

5 | CONCLUSION

FOCI can directly visualize essential structural epidermal changes in atopic eczema *in vivo* in real-time and with cellular resolution, without the need of taking biopsies, which disturbs the natural state of the afflicted skin. FOCI is applicable to non-intervention serial study of precisely the same site in the skin, in lesional skin, and in a control site.

FOCI can be used to assess acanthosis, spongiosis, and parakeratosis of the epidermis supplemented with morphometric study of individual cells of the epidermis particularly the keratinocyte, *in situ*.

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