Novel approaches to characterize age-related remodelling of the dermal-epidermal junction in 2D, 3D and in vivo

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Background/purpose: The dermal-epidermal junction (DEJ) forms epidermal protrusions down into the dermis (rete ridges) and dermal projections up into the epidermis (dermal papillae). Usually visualized in two-dimensions (2D), our knowledge of how the DEJ changes with ageing is limited. We aimed to characterize how this structure exists in 3D and changes with age.

Methods: Photoprotected and photoexposed skin were imaged using reflectance confocal microscopy (RCM) in young and aged individuals. Biopsies of the imaged areas were processed for histological sectioning and for imaging using micro-computed X-ray tomography (microCT).

Results: Images obtained from RCM and microCT were used to 3D reconstruct the DEJ. DEJ heights obtained from microCT images showed strong correlation with histology-measured heights. We proposed a novel definition of rete ridges (RRm) and dermal papillae (DPm), which allowed easier automated measurement of reduced DPm and RRm volumes in aged skin from microCT reconstructions. An algorithm to map DPm connectivity showed reduced lengths of DPm branches with age.

Conclusion: Three-dimensional images illustrated the complex topography of the DEJ and highlighted the distinct morphology of dermal papillae compared with rete ridges, which is not evident when evaluating 2D sections. Ex vivo imaging was more successful in differentiating DEJ architecture with respect to age.

Key words: skin ageing – X-ray computed tomography – reflectance confocal microscopy – dermal-epidermal junction – photodamage – rete ridge – dermal papilla

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As we age, both the appearance and composition of the skin change (1). These changes in appearance are driven by molecular changes within the skin, such as alterations in the architecture of the collagen and elastic fibre network and a flattening of the dermal-epidermal junction (DEJ), which separates the epidermal and dermal compartments of the skin (2). The severity of these changes is often dictated by whether the skin site has been repeatedly exposed to environmental factors (primarily sunlight) or in the main protected, and so acted upon primarily by inherent genetic changes, which occur with the passing of time (3). These two types of ageing are referred to as extrinsic ageing, (or specially photoageing, if referring to ageing induced by sunlight) and intrinsic ageing respectively (4). Although a well-documented feature of both intrinsic and extrinsic skin ageing, changes to the structure of the DEJ are more evident in photoexposed sites (5), but are still relatively poorly understood. However, flattening of the DEJ is thought to have important functional consequences, as it compromises the adhesion of the epidermis by way of the
downward projecting rete ridges interlocking with the upwardly protruding dermal papillae of the dermis. This flattening also reduces the surface area over which nutrients, cells and signalling molecules may pass into the avascular epidermis from the dermis (6). However, despite the three-dimensional (3D) structure of the DEJ being integral to its function, attempts to characterize age-associated changes to this structure have thus far been mainly limited to two-dimensional (2D) histological investigations.

Use of histology for structural investigation of the skin is a relatively simple and cheap technique using equipment that is readily available in most laboratories and can provide compositional information if used in tandem with various labelling techniques (7). However, this requires the fixation, sectioning and then staining of the skin, a multistep process, with each stage adding a number of artefacts to the skin sample. Final visualization of the DEJ and the skin as a whole, after processing, may therefore be somewhat removed from how the skin actually existed in the body and only provides a 2D image.

Micro-computed X-ray tomography (microCT) can, in part, offer a solution to some of these histology-related issues. MicroCT has been used extensively to image and model structures in 3D, but biological applications have in the main been limited to investigation of bone and other calcified tissues (8). This is in part due to the lower X-ray contrast of non-calcified biomaterials (9), although lately microCT has successfully been used to image human skin (10). Resolutions comparable to optical microscopy can now be obtained from whole biopsy specimens, thereby circumventing possible tearing and damaging of delicate structures (11). However, this technique still requires a biopsy to be taken, and in most cases, fixation and dehydration of the tissue. Image acquisition is also often lengthy and so by contrast makes attractive the use of \textit{in vivo} techniques for skin imaging, which are rapid, allow longitudinal monitoring and avoid specimen preparation artefacts.

The use of \textit{in vivo} techniques for examining the skin has increased vastly in recent years, with many different techniques now available (12). Ultrasound is generally the technique of choice when investigating large areas of skin and/or deeper into the skin tissue, but even modern high-resolution ultrasound instruments are limited by low resolution (13). Optical coherence tomography (OCT) is a technology similar to ultrasound, which uses near-infrared light rather than sound to produce images. Using OCT, higher spatial resolution can be obtained with a maximal penetration depth of approximately 2 mm (14), although for some applications, it too can lack sufficient resolution (15), especially if trying to investigate substructures within the epidermis (16). Multiphoton excitation spectroscopy, second/third harmonic generation and Raman spectroscopy are newer technologies belonging to the family of imaging techniques known as non-linear optical spectroscopy (17). These techniques can allow relatively high-resolution visualization of the epidermis and give insight into chemical composition (18, 19). However, their rate of image capture can be slow, and in some cases, sensitivity when imaging the skin may be low (20, 21). Reflectance confocal microscopy (RCM) provides excellent lateral resolution with which to visualize the skin down to the papillary dermis \textit{in vivo} (22). RCM uses a laser emitting diode of near-infrared light to provide high-resolution images captured at relatively fast speeds by virtue of the differing refractive indexes of skin structures (23). Although no information on chemical composition is provided by RCM, availability and use of these microscopes is becoming more widespread as they are approved and used in dermatology clinics for the investigation of possible skin cancers and other dermatological diseases (24).

Previous studies have used RCM to characterize ageing in the skin. However, they have not, to the best of our knowledge, used 3D reconstructions to compare features of intrinsic ageing and photoageing of the epidermis and DEJ in tandem with microCT. Further, with the preparation of histology sections from the same area of skin, direct comparison with the gold-standard technique of histology is possible, giving information as to the accuracy and reliability of measurements taken using these different imaging techniques. The aim of this study therefore was to compare the ability of \textit{in vivo} (RCM) and \textit{ex vivo} (microCT and histology) methods to visualize and quantify key aspects of skin epidermal and DEJ morphology in 3D and determine whether these measurements could enhance our understanding of DEJ remodelling as a consequence of ageing.
Methods

Volunteers
Volunteers aged 18–30 years (mean: 24 ± 3 years) and 65 years or over (mean: 71 ± 4 years) were recruited into the study (n = 10 per group; six females per group; University of Manchester Research Ethics Committee ref 13268). All subjects gave written, informed consent and the study was carried out in accordance with the declaration of Helsinki guidelines (1996). Study subjects were Fitzpatrick skin phototype I-III.

In vivo imaging and biopsy removal
An area of skin on the mid dorsal forearm (photoexposed) was imaged using a RCM (VivaScope® 1500; MAVIG GmbH, Munich, Germany); five stacks of sixty-five 500 μm × 500 μm images were taken at 3.05 μm intervals to give skin ‘stacks’. RCM was also performed on an area of the photoprotected upper buttock/hip/lower back. Once imaging was complete, biopsies (6 mm) were taken of the imaged areas under local anaesthesia and immediately fixed in Bouin’s fixative for 4 h. Following fixation, the biopsies were bisected, with one portion prepared for histology and the other for microCT imaging.

Histology
After fixation, the biopsies were serially dehydrated, cleared and wax-embedded. Wax sections of 4 μm in thickness were slide-mounted and then dewaxed and hydrated before haematoxylin and eosin (H&E) staining. Stained sections were dehydrated and mounted using DPX mounting media (Sigma-Aldrich, Gillingham, UK). Sections were imaged using All-in-one Type Fluorescence Microscope Biozero-8000 (Keyence, Osaka, Japan).

MicroCT
Following fixation, the biopsies were stained in Lugol’s solution [I₂KI; 1% I₂, 2% KI in water; diluted to 10% in water immediately prior to staining as described by Metscher (25)] for 18 h before rapid dehydration (to minimize stain loss) followed by clearing and wax embedding. Biopsies were wax-embedded using a 200 μL pipette tip as a mould to both minimize the volume of wax surrounding the biopsy and create a smooth rounded surface with no sharp edges. Stained, embedded biopsy samples were imaged with a Carl Zeiss Xradia Versa-510 system (Carl Zeiss XRM, Pleasanton, CA, USA) using the 4× objective with a source voltage and current of 50 kV and 80 μA respectively. The source and detector were positioned ~9 mm and 20 mm, respectively, from the sample, resulting in a voxel size of 1.05 μm. Tomographic reconstruction was carried out using the Feldkamp, Davis, Kress (FDK) algorithm, and the resulting images were segmented in AVizo 8 software (FEI Visualization Sciences Group, Hillsborough, OR, USA) using a conventional thresholding approach, which relied on differences in X-ray density.

Image analysis
Histology
DEJ/rete ridge height was measured using H&E-stained sections and was measured from level with the DEJ in the adjacent non-rete ridge region of the skin to the base of the rete ridge for all rete ridges visible on a section [see Fig. 5a(i) for illustration]. Three sections were analysed per person per body site.

MicroCT
Using microCT reconstructed images, the stratum corneum was segmented in three non-overlapping regions of interest (of 260 μm³ volume) within each skin biopsy. The thicknesses of the resulting segmentations were then calculated using the local thickness plugin in ImageJ (26, 27). A mean value was calculated from the three segmentations. DEJ/rete ridge height was determined by segmentation of the epidermis in two non-overlapping regions of interest, each with a volume of 470 μm³, within each skin biopsy. The low frequency curvature of the epidermis, which is apparent in Fig. 2a(i), was then removed by fitting a smooth surface to the overlying stratum corneum. The epidermis segmentation was then flattened with respect to this surface by resampling the data along the normals to the surface. Peaks and troughs in the DEJ were then identified by finding the local maxima and minima in the height of the DEJ within a sliding window having a diameter of 25 pixels (i.e. approximately large enough to
include the peak and trough of a rete ridge within that area). The DEJ/rete ridge heights were calculated as the difference between the local maxima and minima.

Reflectance confocal microscopy

Reflectance confocal microscopy stacks were subjected to segmentation to separate the dermis from the epidermis in order to reconstruct the surface corresponding to the DEJ. This was a semi-automatic segmentation method that required an operator to manually label dermal and epidermal compartments to initialize the algorithm. The 3D images were then automatically pre-segmented into superpixels with homogeneous grey level properties, and classified into dermal and epidermal compartments using a Graph-Cut approach. This process worked in real-time and allowed a trained user to segment the DEJ of the confocal stack images in 10 min. The dermal papillae were segmented from four stacks per person.

Stratum corneum thickness using RCM images was determined by plotting mean grey-scale values from each image down the stack and designating the start of the stratum corneum to be the mid value of the grey-scale curve increase from entering the stratum corneum. The end of the stratum corneum was taken to be the first appearance of stratum granulosum cells, as used by Böhling et al. (28). The thickness was then determined by the number of stack images between the start and end image, with each image taken 3.05 \( \mu m \) apart. Four to six stacks were analysed per person per body site.

Manually derived DEJ/dermal papillae height was determined similarly by noting the start and end image for each whole papilla visible in a stack and then a mean papillae height calculated for each stack [see Fig. 5a(i) for illustration]. Any papillae only partially visible due to being located around the edge of the stack were not analysed. Four stacks were analysed per person.

Dermal papillae (DP\(_m\)) and rete ridge (RR\(_m\)) volume and network analysis from reflectance confocal microscopy and microCT 3D reconstructions

Structures within the region occupied by the DEJ were first defined as either DP\(_m\) or RR\(_m\) by the calculation of a median plane through the DEJ region. Dermis above this median plane was designated as DP\(_m\) and epidermis below this median plane was defined as RR\(_m\). The median plane was computed using a second order polynomial regression of the surface delimiting epidermis and dermis in order to remove any low frequency curvature of the epidermis. DP\(_m\) and RR\(_m\) heights were calculated by measuring the distance between each point of the DEJ surface and the median plane. The volume of these structures was computed by summing the volumes encapsulated between the median plane and the DEJ surface above the plane for DP\(_m\) and under the plane for RR\(_m\) [see Fig. 5a(ii) for illustration].

DP\(_m\) networks were created from the microCT 3D reconstructions and DP\(_m\) designated by the calculation of a median plane, as described above. DP\(_m\) networks were generated by identifying the number of isolated DP\(_m\) ramifications/branches at each distance (pixel) from the median plane; that is, at each distance/row of pixels from the median plane upwards, each pixel was assigned as a DP\(_m\) or not a DP\(_m\). Each point designated a DP\(_m\) was then linked to pixel rows above and below similarly assigned as a DP\(_m\) to create the connectivity between each ramification/branch of all the DP\(_m\) within a skin segmentation. Network graphs (Fig. 7a) represent the structure and branching of all the DP\(_m\) within a segmentation flattened into a single 2D image; that is, each DP\(_m\) starting from the median plane and dividing into ramifications/branches as they move up into the epidermis. The longer the ramifications/branches are, the bluer they appear in the graphs.

Statistics

All image analysis was carried out blinded. Where data were parametric, mean and standard deviation are displayed and subsequently analysed using Student’s unpaired \( t \)-test or two-way ANOVA. Non-parametric data, or where the \( n \) number was 4 or fewer, were subjected to a Mann–Whitney test and displayed as medians with interquartile range (IQR). Distributions were compared using a two-sample Kolmogorov–Smirnov test. Mean and median values obtained using different imaging modalities were compared using a Pearson’s \( r \) correlation test.

Results

Using standard histological methods for comparison, this study aimed to determine if ex vivo
(microCT) and in vivo (RCM) 3D methods were capable of: (i) visualizing the DEJ and (ii) enhancing our understanding of the epidermal/dermal interface in young and aged, photoprotected and photoexposed skin.

Dermal-epidermal junction structure was characterized histologically by manually measuring rete ridge height. In aged skin (both forearm and buttock), rete ridge height was significantly less than in young skin (forearm; young: $37.6 \pm 6.9 \mu m$, aged: $26.2 \pm 4.5 \mu m$, $P < 0.05$: buttock; young: $61.7 \pm 13.2 \mu m$, aged: $46.0 \pm 15.3 \mu m$, $P < 0.01$) [Fig. 1a(ii)]. However, there was no significant alteration in the number of rete ridges in aged skin compared with young either in the buttock (young: 10 per mm ± 1, aged: 10 per mm ± 3) or the forearm (young: 6 per mm ± 2, aged: 5 per mm ± 3) [Fig 1a(iii)].

To be able to visualize the DEJ in our biopsy samples, not just in 2D, but in 3D we employed microCT imaging. With the aid of an exogenous X-ray contrast agent we visualized many skin structures including hairs, epidermal furrows and even the honeycombed patterning of individual keratinocytes (Fig. 2). Segmentation of the epidermis and stratum corneum was also possible. Segmentation of the stratum corneum allowed measurement of the thickness of the stratum corneum ex vivo, which is not reliable if measured on histological sections due to the stretching and tearing caused by sectioning. No difference in the thickness of the stratum corneum was measured between skin from young (young buttock: 11.3 µm IQR 2.8, young forearm: 13.6 µm IQR 3.9) or aged (aged buttock: 10.7 µm IQR 2.8, aged forearm: 12.4 µm IQR 6.5) participants at either body site (Fig. 3a) and

Fig. 1. A reduction in DEJ/rete ridge height can be detected using manually measured histological images. (a) Representative images of H&E-stained histological sections (i) A reduction in DEJ/rete ridge height with age both in the photoprotected buttock and photoexposed dorsal forearm was found in our cohort. There was also a difference in heights between the photoprotected buttock and photoexposed forearm (young vs. aged, $P < 0.001$ & buttock vs. forearm, $P < 0.001$ overall in a 2-way ANOVA, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Sidak’s post hoc tests; mean values with SD are displayed; $n = 10$; (ii)]. There was no difference in the number of rete ridges measured in skin from aged individuals compared with young, although there were fewer rete ridges present in the forearm compared with the buttock (buttock vs. forearm, $P < 0.001$ overall in a 2-way ANOVA, **$P < 0.01$, ***$P < 0.001$, in Sidak’s post hoc tests; mean values with SD are displayed; $n = 10$; (iii)]. Scale bars represent 100 µm.
Fig. 2. Three-dimensional reconstructions of whole skin biopsies may be created from microCT images. (a) 3D reconstructions of microCT imaged skin biopsies stained with I$_2$KI allowed visualization of a number of structures within the skin. View of the epidermis from above (stratum corneum not shown); the honeycomb pattern of individual keratinocytes can be visualized in the epidermis, as can the furrow pattern (i). Whole biopsies may also be visualized in any orientation, including from the bottom/side, where the larger dermal region is visible compared with the narrower epidermal region above. The epidermis is more X-ray dense than the dermis and so appears lighter. Skin appendages such as hair follicles can also be seen (ii). Virtual sectioning through the biopsy allowed visualization of skin biopsies in cross section and the identification of structures on the inside of the biopsy including eccrine glands (iii) and vessels (iv). Scale bars represent 100 μm.

Fig. 3. The stratum corneum and epidermis can be readily segmented from microCT reconstructions. Using differences in X-ray density key skin structures were segmented. (a) Segmentation of the stratum corneum and subsequent thickness calculations revealed no difference in stratum corneum thickness with age [Mann–Whitney, median values with IQR are displayed; n = 66–7; (i)]. (b) Segmentation of the epidermis allowed visualization of the complex nature of the DEJ and how this differs with body site and age. The segmented epidermis are shown side-on with the skin surface/stratum corneum above and DEJ surface below. These have then been rotated 90° to show the DEJ surface facing forwards. Scale bar represents 100 μm (i). Using these segmentations to plot a histogram of the distribution of DEJ/rete ridge heights (ii) and the cumulative frequency distributions (iii) showed a significant alteration with age both in the buttock (D = 0.47, P < 0.001), and forearm (D = 0.48, P < 0.001). Two-sample Kolmogorov–Smirnov test; cumulative frequency distributions are displayed; n = 48–5 per group compiled into each distribution. Median DEJ/rete ridge heights obtained from microCT segmentations showed good correlation with values obtained from histological sections (iv); r = 0.86, P < 0.0001, Pearson’s r correlation; n = 16; solid line represents the line of regression and dotted line marks equal height values. (a) Represents volumes of 260 μm$^3$, b (i) volumes of 470 μm$^3$. 
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(a) Stratum corneum thickness (μm)

(b) (i) Stratum corneum surface and DEJ surface

(ii) Histogram

(iii) Cumulative frequency distribution

(iv) Rete ridge height (μm)
agreed with measurements from the RCM, which also showed no difference (data not shown).

Segmentation of volumes of the epidermis from microCT-imaged skin biopsies allowed visualization of the complexity of the topography of the DEJ and the changes with age, which were more dramatic in the photoexposed skin [Fig. 3b(i)]. Analysis of the DEJ by way of the rete ridges segmented from microCT images showed a shift in the distribution of buttock skin DEJ/rete ridge heights with age [Fig. 3b(ii)]. Approximately 50% of rete ridges were at or below 50 µm in the aged buttock skin compared with approximately 75 µm in the young \(P < 0.001; \text{Fig. 3b(iii)}\). In the forearm, DEJ/rete ridge heights were both reduced in the skin from aged volunteers, but also showed a reduced spread, as illustrated by a narrower peak in the histogram and a steeper line gradient in the cumulative frequency distribution compared with the young \(P < 0.001; \text{Fig. 3b(iii)}\). Interestingly, aged buttock and young forearm were very similar in terms of their DEJ/rete ridge height profiles [Fig. 3b(ii) & (iii)]. Comparison of the DEJ/rete ridge heights from these 3D reconstructions and subsequent analyses were then compared with 2D histology as a way to validate this analysis technique. Good correlation of microCT DEJ/rete ridge height values were obtained when compared with values measured from histological sections. This demonstrated that microCT imaging, segmenting and using local maxima and minima height profiles to determine DEJ/rete ridge heights compared well with histology \(r = 0.86, P < 0.0001; \text{Fig. 3b(iv)}\). Next, we determined if in vivo imaging techniques were able to provide similarly robust characterization of the DEJ.

Reflectance confocal microscopy imaging has been used to investigate the skin in vivo for many applications, including ageing (29, 30), but use of these images for skin and DEJ visualization in 3D remains underdeveloped. However, we began by carrying out a manual comparison of the DEJ by way of measuring dermal papillae (which are easier to visualize than rete ridges when using RCM) heights and number to compare with histology. Dermal papilla height could not effectively be determined in some RCM stacks, especially those from the buttock due to a lack of dermal papillae definition/contrast/melanin in this cohort [Fig. 4a(i) vs. (ii)]. Using images where dermal papillae could be sufficiently differentiated, there was not a significant reduction between aged and young buttock (aged: 42.4 µm IQR 12.9, young: 54.7 µm IQR 15.9) or aged and young forearm skin (young: 47.3 µm IQR 14.5, aged 38.3 µm IQR 18.5; Fig. 4a(iii)]. Similar to histological findings, no significant difference was found in the number of dermal papillae observed in young compared with aged skin either in the buttock (young: 54 per mm² IQR 35, aged: 37 per mm² IQR 56) or forearm (young: 48 per mm² IQR 20, aged: 27 per mm² IQR 58; Fig. 4a(iv)]. Comparison of DEJ/dermal papillae heights obtained using RCM with histology, (taking histology to be the gold-standard method) showed that RCM measurements tended to over-estimate shorter dermal papillae and under-estimate the taller (Fig. 4b).

Manual analysis of dermal papillae heights was therefore not as effective at differentiating the shorter and taller dermal papillae and did not provide 3D information. As a result we developed a computational method of dissecting out dermal papillae from the RCM images in order to better understand how this structure exists in situ. Development of a semi-automatic, near real-time segmentation program allowed the creation of the DEJ/dermal papillae in 3D from RCM stack images taken of forearm skin of young individuals. These images provided clear, relatively high-resolution reconstruction of the DEJ/dermal papillae in vivo (Fig. 4c).

Fig. 4. No significant difference in DEJ/dermal papillae height was measured between young and aged skin using RCM images, but 3D reconstructions of the DEJ/dermal papillae could be created. (a) Stack images show examples where dermal papillae were highly distinct (i) and very difficult to distinguish (ii). Including only those individuals where dermal papillae were sufficiently distinct to be differentiated by eye, there was no significant reduction in dermal papillae height with age, (young buttock vs. aged buttock and young forearm vs. aged forearm, Mann–Whitney test; median values with IQR are displayed; \(n = 5–9\); (iii)). The number of dermal papillae per area also did not alter with age when measured using RCM images [Mann–Whitney test; median values with IQR are displayed; \(n = 5–9\); (iv)]. (b) Comparison of DEJ/dermal papillae heights obtained using RCM images with heights obtained from histological sections showed that shorter dermal papillae tended to be over-estimated and taller dermal papillae under-estimated \(r = 0.63, P < 0.001, \text{Pearson’s } r\) correlation; \(n = 29\); solid line represents the line of regression and dotted line marks equal height values). (c) Example image of a 3D reconstruction of the DEJ/dermal papillae in high resolution produced from RCM stack images of forearm skin. Scale bars represent 100 µm.
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(a) and (ii) Images showing the structure of the dermo-epidermal junction (DEJ) in young and aged skin.

(iii) Graphs showing the comparison of dermal papilla height and number of rete ridges/mm² between young and aged skin on different body parts: buttock and forearm.

(b) Scatter plot showing the relationship between dermal papilla height (histology: μm) and rete ridge height (CM: μm), with a correlation coefficient of $r = 0.63$. $***P < 0.001$.

(c) 3D image depicting the dermal papilla and furrow structures.
Although 3D reconstructions of RCM images were greatly advantageous in giving insight into the DEJ as it exists in the body, lack of contrast of dermal papillae due to low levels of melanin in our cohort meant 3D reconstructions could only be performed from images taken from the photoexposed forearm site. However, we also wanted to extend our analysis parameters and measure not only DEJ height which, after all, is possible on histological sections, but use analysis techniques to quantify the 3D topography of the DEJ. Due to the undulating nature of the DEJ and difficulties in defining the top and bottom of individual rete ridges and dermal papillae for use in automated computer algorithms, a new approach was used (Fig. 5a). This found a horizontal median plane through the region of skin occupied by the DEJ and designated dermis above that plane DPm [Fig. 5b(i)] and epidermis below it as RRm [Fig. 5b(ii)]. Distinction of the DPm and RRm using a median plane more clearly illustrated dermal papillae as discrete protrusions [Fig. 5b(i)] in contrast to the interlinking network of rete ridges [Fig. 5b(ii)].

Using this model applied to the 3D reconstructions (Fig. 6a), we could then make volume measurements on both RCM and microCT forearm data. While a significant reduction in the volumes of both DPm (young: 21.0 × 10^5 µm^3 IQR 12.1, aged: 12.2 × 10^5 µm^3 IQR 3.0) and RRm (young: 24.3 × 10^5 µm^3 IQR 14.2, aged: 12.4 × 10^5 µm^3 IQR 4.3) was found with age using microCT images (P < 0.05; Fig. 6b), this difference was not measured when analysing RCM 3D images (DPm, young: 0.92 × 10^5 µm^3 ± 0.19, aged: 0.80 × 10^5 µm^3 ± 0.19; RRm, young: 2.02 × 10^5 µm^3 ± 0.93, aged: 2.30 × 10^5 µm^3 ± 1.5; Fig. 6b).

In our hands and with our study cohort, microCT images provided better quality data with which to compare DEJ structure in young and aged individuals. We wanted to further explore the connectivity of dermal papillae, so using the same median plane technique, we used microCT images to compare connectivity of DPm in the photoprotected buttock with the photoexposed forearm (Fig. 7a). Networks represent the connectivity path taken by each DPm beginning at the median plane and moving upwards into the epidermis over the whole 3D segmentation and has then been flattened to enable viewing of all DPm within the segmentation on just two axes. More convoluted DEJs would therefore be represented by more and/or longer branches. These network trees were then used to calculate the distance of each branch from the median plane and the length of each branch within the tree, with the more blue the branch, the longer the length. Young buttock and forearm DPm were of largest volume further from the median plane (Fig. 7b). Interestingly, however, buttock and forearm sites did not differ greatly in either the distance of their branches from the median plane [7c(i)] or branch length [Fig. 7c(ii)]. The major difference was instead between networks in young and aged skin in relation to the distance of each branch from the median plane [Fig. 7d(ii)], with the young skin possessing more branches penetrating further up into the epidermis (buttock: 20.6 µm IQR 5.8; forearm: 21.1 µm IQR 7.5) than aged (buttock: 13.3 µm IQR 3.1; forearm: 13.1 µm IQR 4.0). Branch length was not significantly shorter in young skin compared with aged at the same body site [aged buttock: 4.8 µm IQR 1.4; aged forearm: 3.8 µm IQR 1.4; young buttock: 7.6 µm IQR 3.3; young forearm: 5.2 µm IQR 3.8; Fig. 7d(ii)].

**Discussion**

In this study we wished to compare the ability of *in vivo* and *ex vivo* techniques to quantify aspects of DEJ morphology and to determine if 3D imaging modalities (microCT and RCM) could visualize and quantify the 3D topography of the DEJ in intrinsically and extrinsically aged skin. Further to this, we also wished to determine if these approaches would be able to detect subtle changes in the epidermis, as other studies have suggested that *in vivo* imaging approaches might be able to negate the need for skin biopsies in future (14, 31, 32).

Using microCT to image skin treated with a contrast agent, we have shown that numerous skin structures can be visualized and in fine detail. Although still requiring a biopsy to be taken, and in the main slow imaging speeds and use of this technique being expensive, microCT allows us to clearly visualize the complex nature of the skin at high resolution. As sectioning is not required, tears and artefacts that are often associated with sectioning can be avoided and the biopsy remains whole, allowing for further use of the tissue (10). We have
also demonstrated, we believe for the first time, that it is possible to segment stratum corneum and epidermis from microCT-imaged skin samples. Stratum corneum thickness values in our study obtained by microCT are similar to those reported in other studies (20, 28, 33–37).

Fig. 5. Dermal papillae and rete ridges may be more easily defined by using a median plane (DPm & RRm), when using automated 3D structural analysis. (a) Depiction of rete ridges and dermal papillae heights as they are usually defined (i). However, we proposed an alternative where a median plane through the DEJ region was determined and dermis above this median plane designated a DPm (blue) and epidermis below this median plane assigned as a RRm (orange) (ii). (b) This analysis method was applied to both CM and microCT 3D reconstructions. Use of a median plane also highlighted the discrete pillar-like structure of dermal papillae (i) as opposed to the long interconnected nature of rete ridges (ii). Both DPm and RRm can be depicted on the same segmentation (iii). Images show volumes of skin of 470 μm³ and scale bar represents 100 μm.
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(a) Young forearm

(b) DP_m volume

RCM

Volume (1x10^5 μm^3)

Young  |  Aged
-----  |-----
0.8    | 0.6  

MicroCT

Volume (1.1x10^5 μm^3)

Young  |  Aged
-----  |-----
30   | 20  

RR_m volume

Volume (1x10^5 μm^3)

Young  |  Aged
-----  |-----
4.0    | 3.0  

*
but lower than some in vivo measured values (38, 39). DEJ height values obtained from microCT-imaged segmented epidermises were well matched with manually measured histological values and so, once segmented, this technique provides a much more rapid way to measure DEJ height over a relatively large volume of skin and with high accuracy. It also provided clear illustrations of the topography of the DEJ and how loss of this topography is enhanced with ageing in photoexposed skin. MicroCT imaging of the skin not only provides high-resolution 3D images of the epidermis, but also of the dermis, making it a useful tool for investigating skin to depths not possible with RCM, OCT and multiphoton due to optical scattering (40–42). Therefore, although not investigated in this study, microCT could be used in future to visualize and study dermal structures. Similar 3D reconstructions of skin biopsies are also possible using high-resolution episcopic microscopy, but compared with microCT has the major disadvantage of requiring physical sectioning after each image is taken, as well as requiring the use of a histological stain (43). Improvements are continually being made in microCT instrumentation, including the use of phase contrast and also spectral CTs. This coupled with improvements in reconstruction algorithms, as well as at the more specialist end of this technique, the use of synchrotron-produced X-rays, means that in future the use of contrast enhancers will not be necessary for all soft tissue imaging and better resolutions obtained with more rapid image acquisition times (11, 44).

RCM has been used in many in vivo investigations of the epidermis and DEJ. However, rather than using these images to score structures by visual assessment of their appearance (29, 45, 46), we sort to extract quantitative height, volumetric and connectivity data on the DEJ. Use of a manual method to extract height data proved difficult in some individuals and most particularly in the buttock, as a result of not being able to visualize papillae in this photoprotected site due to low levels of pigmentation. This coupled with the reduced accuracy of height measurements compared with histology meant that a significant reduction in DEJ/dermal papillae height was not detected, even though from histology we had measured a significant reduction in this cohort. Measurements using RCM in this case were less accurate than when using histology, as histology images can be measured to the nearest pixel (0.65 μm in our case), but RCM resolution limits images to a useful minimum lateral image distance of 3.05 μm. This lesser resolution in the Z-axis together with a maximal imaging depth of approximately 150–200 μm often makes the end of dermal papillae particularly difficult to distinguish. In this way, using RCM to measure DEJ height can be a blunt instrument compared with ex vivo techniques. Analyses of dermal papillae height in ageing skin using RCM have been carried out in previous studies, although these have often used darker skinned individuals (45, 47), as it has been shown that dermal papillae contrast is linked with skin pigmentation (22, 48). Higher concentrations of fibrillin/oxytalan fibres present within in the dermal papillae might also further reduce dermal papillae contrast, as would make the interior of the dermal papillae appear less dark. Therefore, the use of study volunteers Fitzpatrick type III and above provides images containing dermal papillae that are more defined. Increased melanin is also more likely to be present in photoexposed areas, making RCM a better choice for investigation of the DEJ at these sites.

In our cohort we found no significant reduction in the number of dermal papilla with age either in the photoexposed or photoprotected site. A study using RCM to investigate ageing in a Japanese population did record fewer dermal papillae in aged forearm skin at a depth of 50 μm, but no difference in the number of papillae at 80 μm depth (47). A decrease in papillae number with age and/or sun exposure

Fig. 6. DPm and RRm volumes are reduced in photoexposed aged skin segmentations created from microCT images compared with young. (a) Example images of young and aged forearm DEJ topography segmented from RCM and microCT images. Our definition of rete ridge and dermal papilla was applied as defined in Fig. 5 so that the dermis above the median plane are DPm shown in blue and epidermis below the median plane RRm and coloured orange. RCM images represent segmentations from stacks of images 500 μm x 500 μm in area, microCT segmentations represent volumes of skin of 470 μm^3, scale bars represents 100 μm. (b) Volume calculations of RRm and DPm from these images showed that a difference in volume cannot be measured between RCM reconstructions of young compared with aged skin. However, a significant difference was found between young and aged when comparing DPm and RRm volumes measured from microCT reconstructions (*P < 0.05, Mann–Whitney test; n = 48±5).
has been measured in Fitzpatrick skin phototype I-III cohorts using RCM (30, 48, 49). Therefore, although the finding of no alteration in dermal papillae number is not similar to other published results, it does match the histology data from our cohort showing no significant difference in the number of rete ridges between young and aged skin. A more robust measure of papillae number in fair skin using RCM may have been to take videos to capture blood flow and thereby deduce the presence of a dermal papilla, as recently used by Haytoglu et al. (49).

We then went on to use a novel near-realtime semi-automated method to segment dermal papillae from RCM images and reconstruct the DEJ. This allowed 3D visualization and quantitative characterization of the DEJ without the artefacts caused by biopsy removal and tissue fixation. Three-dimensional reconstructions of dermal papillae at lower resolutions have been carried out using images from multi-harmonic generation microscopy (50), although at lower resolutions and smaller lateral areas compared with those in our study. Higher resolution images of the DEJ taken from RCM images have been produced by the Rajadhyaksha lab (51), although like us, they found DEJ differentiation in images from skin phototypes I and II challenging. As a result, Kurugol et al. (51) produced both a dermal profile and an epidermal profile with a ‘transition zone’ between the two. However, for our subsequent measurements, we required a consensus DEJ and so excluded those images where the algorithm could not differentiate dermal papillae.

In this study, we proposed a novel method of quantitatively assessing the 3D nature of the DEJ. Our assignment of DP\textsubscript{m} and RR\textsubscript{m} by way of a median plane through the volume occupied by the DEJ was a technique that could be used both on RCM and microCT 3D reconstructions. The main limitation of this analysis technique was that full heights and volumes of the rete ridges and dermal papillae were not calculated, as structures that would classically be classified as part of a rete ridge or dermal papilla, but fall above (for rete ridges) or below (for dermal papillae) the median line were not considered. However, this technique overcame the difficulty of having to assign a top and a bottom for each individual dermal papilla and rete ridge, which is ever changing as one moves across a sample. The higher clarity with which the DEJ could be resolved from microCT images resulted in superior 3D reconstructions. This is likely to be the reason for a significant reduction being measured in the volume of DP\textsubscript{m} and RR\textsubscript{m} in aged skin compared with young using microCT, but not RCM images. That being said, we cannot rule out the possibility that, in vivo, differences in volume are not so large between young and aged skin. Removal of the skin so it is no longer under tension and the process of dehydration may exacerbate length and volumetric differences, as neither did we find significant reductions in dermal papillae height using manual measuring of RCM images. Indeed, using images from harmonic generating microscopy Liao et al. (50) found no alterations in dermal papillae volume with age. However, this study was performed in an Asian population, which might experience lesser skin photoageing compared with a Caucasian population (52, 53).

Our results highlight the complex nature of the interconnectivity of the DP\textsubscript{m} network and suggests that in future, we may need to better describe what we mean by a ‘dermal papilla’. Is it a single branch or is it the whole tree network which stems from a single ‘trunk’? Could some topical applications or diseases differentially affect specific sub-populations of dermal
papillae? In this study, we found a greater difference in the DPm network with age, rather than body site. A decrease in 3D DEJ interdigitation and a reduction in the average height of isolated dermal papillae has been found previously (50), but their analysis method did not allow them to measure the height/length of all papillae branches, only isolated papillae, which as our network diagrams illustrate, is a small proportion of all dermal papillae. Multiphoton technology has also been used to produce 3D images of the DEJ in response to retinol and retinoic acid, showcasing 3D visualization of the effect of these topical applications (54). Therefore, future in vivo images may allow the same quality of imaging as ex vivo, enabling detection of more subtle changes in skin architecture.

We, to the best of our knowledge, for the first time were able to create high-resolution 3D reconstructions of the DEJ using both RCM and microCT to investigate changes due to ageing (see Table 1 for a technique comparison summary). MicroCT imaging allowed the 3D reconstruction of the DEJ in both photoprotected and photoexposed sites and allowed us to investigate the changes in topography as a result of intrinsic and photoageing. Therefore, in our hands at least, ex vivo and histological techniques remain the best for the detection of more subtle changes to the DEJ and epidermis as a whole, with histology still the most effective, low-cost method of assessing changes in DEJ topography. As a result, studies undertaken in the near future are still likely to require biopsies to be taken, especially when investigating intrinsic ageing or the effect of topical applications. The use of 3D to represent epidermal structures using microCT and RCM images gives new insight into the complexity of the skin and allows studies to analyse the DEJ in terms of volume and 3D connectivity. For these reasons, visualization and analysis of the skin in 3D is likely to become more commonplace in the future as we further address the technical difficulties associated with these techniques in order to give us a more realistic window through which to view the skin.

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Conflict of Interest

DSM Nutritional Products Ltd has approved submission of the manuscript, but has exerted no editorial control of the content. MJS and CEMG state no conflict of interest. VLN is funded by DSM Nutritional Products Ltd, RV is an employee of DSM Nutritional Products Ltd and AVR is consultant to DSM Nutritional Products Ltd. RSB is funded in part by the Zeiss XRM Fellowship programme. REBW is supported by a programme grant from Walgreens Boots Alliance (Nottingham, UK).

| Technique | Biopsy and fixation | Sectioning required? | Epidermal resolution | Speed of initial image acquisition | DEJ visualization affected by skin pigmentation? | 3D? | Speed of 3D image creation and manipulation | Costs |
|-----------|---------------------|----------------------|----------------------|------------------------------------|-----------------------------------------------|-----|---------------------------------------------|-------|
| Histology | Yes                 | Yes                  | High                 | Low                               | No                                           | No  | Low*                                        | Low   |
| RCM       | No                  | No                   | High (x & y), Medium (z) | High                              | Yes                                          | Yes | Medium- purchase; Low-running                  |       |
| MicroCT   | Yes                 | No                   | High                 | Low                               | No                                           | Yes | Low*                                        | High-running |

- Not applicable; *where contrast is sufficiently high.
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