INVESTIGATIVE REPORT

Variation in Epidermal Morphology in Human Skin at Different Body Sites as Measured by Reflectance Confocal Microscopy

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Two methods of estimating stratum corneum thickness using reflectance confocal microscopy were examined, and epidermal thickness measurements at multiple body sites were compared. Measurements of stratum corneum thickness were made using the derivative method, which is based on the rate of change of image intensity as a proxy for keratin concentration, and simple visual analysis of confocal images. To compare epidermal thickness we collected 1491 z-axis stacks of confocal images from 10 body sites in 39 subjects. An artefact associated with the imaging process interfered with the derivative method for stratum corneum thickness, and simple visual analysis is to be preferred. Although some epidermal properties varied by site, the most striking finding was the degree of within-site variation, which accounted for between 50% and 74% of the total variation observed. The majority of this variation was not due to measurement error, and represents genuine topographical irregularity. This fine-scale variation limits the ease of use of reflectance confocal microscopy for quantitative studies of the epidermis and stratum corneum. *Key words: reflectance confocal microscopy; epidermal thickness; epidermal morphology.*

There is a scale to skin structure and hence skin biology that is not served well by techniques such as conventional transmission microscopy and subcellular analyses on the one hand, and macroscopic examination with the naked eye on the other. For instance, we have shown that differences in epidermal or stratum corneum (SC) thickness at different body sites may be important determinants of site variation in sensitivity to ultraviolet radiation (UVR) (1, 2). However, study of these factors is difficult (3); repeated biopsy at various ultraviolet doses and time-points is ethically problematic, and biopsy means that a single area of skin cannot be observed longitudinally, nor can the three-dimensional structure of skin *in vivo* be appreciated. The fact that the epidermis and the components of the epidermis are not flat regular two-dimensional sheets, but irregular undulating three-dimensional structures, is a quality that has relevance to many aspects of skin biology, including percutaneous absorption, UVR-induced carcinogenesis and ageing. The present study examined the use of reflectance confocal microscopy (RCM) to study epidermal morphology, comparing different methods of measuring SC thickness.

RCM has been described as a new gold standard for the measurement of epidermal thickness (3, 4). It provides a non-invasive imaging tool capable of obtaining *en face* images of living tissue *in vivo* (3). It has none of the problems associated with biopsy-obtained samples that become more or less distorted (swelling or shrinkage) during different steps of the preparation procedure (5–8).

The interpretation of RCM images is based largely on pattern recognition and histological knowledge of skin anatomy (4, 9, 10). For SC thickness one manufacturer of reflectance confocal microscopes suitable for use on human skin, Lucid (Rochester, NY, USA), have proposed a method that we refer to as the derivative method (DM) (personal communication, Lucid 2006). This method takes advantage of the differences in refractive index as skin is traversed vertically and uses the position of the greatest changes in optical density to delineate the margins of the SC. The advantages of this method are that it is rapid, less dependent on the operator, and requires less familiarity with cutaneous histology. To our knowledge there are no published attempts to validate this method. We therefore examined this method and compared the results with SC thickness measurements judged by visual analysis. Subsequently we compared epidermal morphology in a range of individuals at multiple body sites.

MATERIALS AND METHODS

Ethics statement

The study was approved by Lothian Ethics Committee (06/S1104/55).

Confocal imaging

RCM was performed using a Vivascope 1500 (Lucid Inc., Henrietta, New York, USA) equipped with a ×30 objective lens with a numerical aperture of 0.9. Medical ultrasound couplant gel (Diagnostic Sonar Ltd, Kirkton Campus, Livingston, W. Lothian) was used as an immersion fluid for the lens. Either

Crodamol STS oil (Lucid Inc.) or plain distilled water (see below) was used between the skin and the ring template to provide good optical contact.

Experiment 1: Comparison of the derivative method and visual analysis for measuring stratum corneum thickness

Image collection. Images were collected from 4 subjects (2 males, 2 females) at 4 separate body sites using both methods. For each site a vertical (z-axis) "stack" of 51 images was taken at 1 μm intervals to a depth of 50 μm (with the exception of the palm, for which images were collected to a depth of 150 μm for visual analysis).

Visual analysis. Laser power was adjusted manually at increasing depths and Crodamol oil was used at the viewing window/ skin interface. SC thickness was regarded as the difference in depth (in μm) between the two images that represented the top and bottom of the SC. In addition to the experimenter, two novices were also asked to make a determination of SC thickness using the parameters previously described. These results were compared with the investigator's own results.

Derivative method. The DM method used water between the viewing window and the skin in order that the surface of the skin was as close as possible to the viewing window. Laser power was fixed for optimum clarity in the upper granular layer, and the stack collected without further adjustment. Stacks of images were imported into Image J (Wayne Rasband, National Institute of Health, USA; http://rsb.info.nih.gov/ij/_Java1.5.0_13). Using Image J the mean brightness of each image in a stack was calculated and a profile plot generated. The first derivative of the data was calculated and a graph of mean intensity (brightness) vs. depth (μm) plotted. The DM is predicated on the fact that as the SC is the most optically dense area within the epidermis, the maximum and minimum derivative of the serial optical densities define its start and end.

Experiment 2: Image collection for epidermal thickness variation

Volunteers. Twenty-one females and 18 males between the ages of 19 and 84 (mean age women 43, men 46) years were opportunistically recruited into the study between the months of October and May. Their Fitzpatrick skin types were; type I $n=2$; type II $n=20$; type III $n=13$; type IV $n=3$; and type V $n=1$. The number of volunteers and confocal images collected at each body site is summarized in Table I. Body sites were also classified into those more sun-exposed and those relatively sun-protected.

Image collection. For each body site at least one vertical (z-axis) stack of 21 images was collected at 5 μm intervals from the skin surface to a depth of at least 100 μm.

Table I. *The number of z-axis stacks of images analysed at each body site*

Body site	Males (n)	Females (n)	z-axis stacks of images analysed (n)
Mid-dorsum of hand	10	15	360
Centre of calf	6	7	278
Outer forearm	5	2	184
Inner forearm	0	3	73
Inner upper arm	2	4	172
Upper back	7	6	261
Upper front torso	1	1	44
Lower front torso	1	1	55
Corner of eye	0	2	34
Temple	1	0	20

VivaBlocks. The Vivascope 1500 is also capable of automatically "mapping" an area of skin on an xy horizontal plane up to 4 mm² in the form of a VivaBlock. For many of the subjects we created VivaBlock scans to examine the variation in thickness within a small area of skin (in this case 3 mm²) within one body site. This provided us with 36 adjacent z-axis stacks from a single 3 mm² skin site without repositioning the microscope or subject.

Inter-site variability. Detailed examination of 4 separate 500 μm square sites on the forearm was performed by placing a 10×10 square line grid over each image in a z-axis stack and examining every 50 μm square (1% of image) individually for cell content. Squares were colour-coded according to epidermal layer content (e.g. SC, granular cell layer). We were therefore able to quantify the percentage area occupied by a particular skin layer in each image at increasing depths.

Analysis

The following measurements were recorded; SC thickness (distance from the surface of the skin to the appearance of the first living cells of the granular layer); granular layer thickness (distance from the bottom of the SC to the first cells of the spinous layer); depth to the top of the dermal papillae (from the surface of the skin); depth to the bottom of the dermal papillae (from the surface of the skin); papillae length (distance to the top of the dermal papillae subtracted from the distance to the bottom of the dermal papillae); and total epidermal thickness (distance from the skin surface to the mean of the depth of the top and bottom of the dermal papillae).

Data were analysed using "R" software (11). Details of the statistical methods are given with the results.

RESULTS

Experiment 1: Derivative method vs. visual analysis

Measurements of SC thickness were taken at 4 sites on 4 persons using visual analysis and the DM. Although thickness values were not significantly different for the back of the hand and the inner and outer forearms, the values for the palm differed widely between the two methods (89 μm visual analysis vs. 7 μm DM). Since it is well established that the SC on the palm is thick we suspected that the result using the DM was spurious due to an artefact. In keeping with this interpretation the same pattern was evident when we measured a homogeneously inert material, such as card. We believe the explanation is as follows.

During normal operation of the microscope there is a bright "flare" of light as the microscope's focus passes through the viewing window to the surface of the skin, and this persists for several microns depth. This flare of light is caused by differences in refractive indexes and field curvature (11–13). Because the derivative method uses a fixed laser power, it is not possible to "turn down" the brightness to compensate for the artefact. It is likely therefore that the DM measures the flare rather than the SC situated deep to the flare and effectively precludes the DM as a means of accurately measuring SC at any site.

Table II. *Mean thickness values for epidermal layers at each body site*

Body site	No. of z-axis stacks analysed	Stratum corneum	Granular layer	Top of dermal papillae	Bottom of dermal papillae	Papillary length	Total epidermal thickness
Back of hand	360	9.3 μm	14.0 μm	42.8 μm	82.4 μm	39.6 μm	62.5 μm
Centre of calf	278	8.7 μm	10.9 μm	40.3 μm	80.7 μm	40.4 μm	60.5 μm
Outer forearm	184	10.9 μm	9.9 μm	43.8 μm	77.0 μm	33.2 μm	60.3 μm
Inner forearm	73	6.2 μm	8.5 μm	38.1 μm	80.6 μm	42.4 μm	59.4 μm
Inner upper arm	172	6.4 μm	8.0 μm	36.4 μm	79.9 μm	43.5 μm	58.2 μm
Upper back	261	8.4 μm	8.1 μm	36.1 μm	75.1 μm	39.0 μm	55.6 μm
Chest	44	6.5 μm	7.6 μm	37.6 μm	74.4 μm	36.8 μm	56.0 μm
Abdomen	55	6.3 μm	8.1 μm	35.9 μm	80.7 μm	50.8 μm	61.3 μm
Corner of eye	34	5.8 μm	7.2 μm	36.6 μm	77.1 μm	40.5 μm	56.8 μm
Temple	20	6.3 μm	9.6 μm	35.0 μm	87.8 μm	52.7 μm	61.4 μm

To assess observer errors in viewing images obtained by visual analysis three experimenters independently assessed the same confocal images and achieved a high level of agreement (± 1 μm for SC and granular layer, and ± 5% for epidermal and papillary thickness).

Experiment 2: Variation in thickness of the epidermis and constituent layers

In total, 31,311 RCM images in the form of 1491 z-axis stacks of confocal images were collected from 10 body sites in 39 subjects. The mean untransformed epidermal layer thickness values of all subjects at each body site are shown in Table II. For purposes of analysis, granular layer thickness and SC thickness were transformed using their reciprocal.

The data was analysed using a mixed effects model, with body site, sex, smoking habits and age treated as fixed variables, and subjects treated as random variables. We were unable to find any explanatory variables that had a significant effect on total epidermal thickness, but mean thickness of SC varied significantly between sites, with the back of the hand, calf, forehead and outer forearm showing thicker SCs. For dermal papillae length both body site and skin type showed a clear relation, with skin type I persons showing thinner papillae (even when the body site was taken into account), and dermal papillae length being shorter on the back of the hand, chest, outer forearm and upper back. We also grouped sites into those more sun-exposed and those relatively sun-protected and compared measurements. The differences were modest and the only statistically significant difference was for papillary length, which was longer in the sun-protected sites ($p = 0.041$).

There was a striking variation at any one body site for a single individual, with within-site variation accounting for 74%, 59%, 49% and 50% of total variation for papillae length, SC, epidermal and granular layer thickness, respectively. The large amount of within-site variation appeared to be due to the complex three-dimensional structure of the epidermis, with even immediately adjacent areas having different dimensions due to rete peg patterns and folding of the upper epidermis. The surface of the skin is scored with a criss-cross pattern of folds and fissures sometimes referred to as "dermatoglyphs". These are clearly seen as dark bands that extend at an angle into the epidermis, occupying as much as 30% of the area of confocal images of the skin surface and can measure 50–100 μm across (Fig. 1). The depth of these markings varies, but can often extend to around 80 μm or more. We were unable to achieve sufficient resolution to image skin underneath the markings, thus the effect on the physical dimensions of the epidermis beneath and immediately adjacent is unclear; for example, whether epidermal layers are compressed underneath or stretched at the sides, as most measurements are therefore inevitably taken from areas that fall in between. All our samples were measured from skin under normal tension, and we do not know if it is possible to "stretch" the skin to mitigate the folding effect of skin markings or how this might affect subsequent thickness measurements, but Corcuff et al. found no significant variation in epidermal thickness with increasing pressure exerted by the surface contact device onto the study area (6).

Detailed examination of four 500 μm square sites on the forearm was possible by placing a 10 × 10 square line grid over each 500 μm square site in a z-axis stack and examining each 50 μm square (1% of image) individually for cell content. This enabled us to plot the
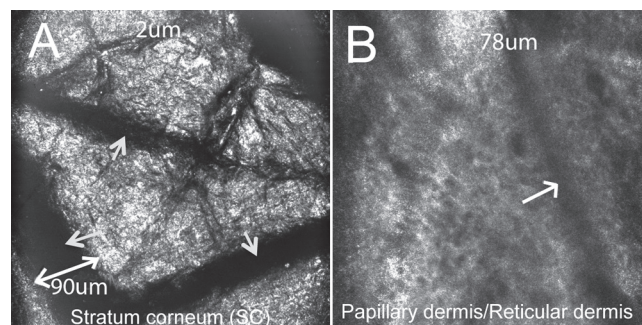

Fig. 1. Skin markings (dermatoglyphs). In confocal images skin markings appear as dark bands that extend into the epidermis at varying angles to the perpendicular. (A) At the skin surface: (→) indicates skin markings, one of the largest measuring 90 μm across. (B) At the papillary dermis (78 μm), image resolution is much reduced: (→) indicates the approaching base of a skin marking.
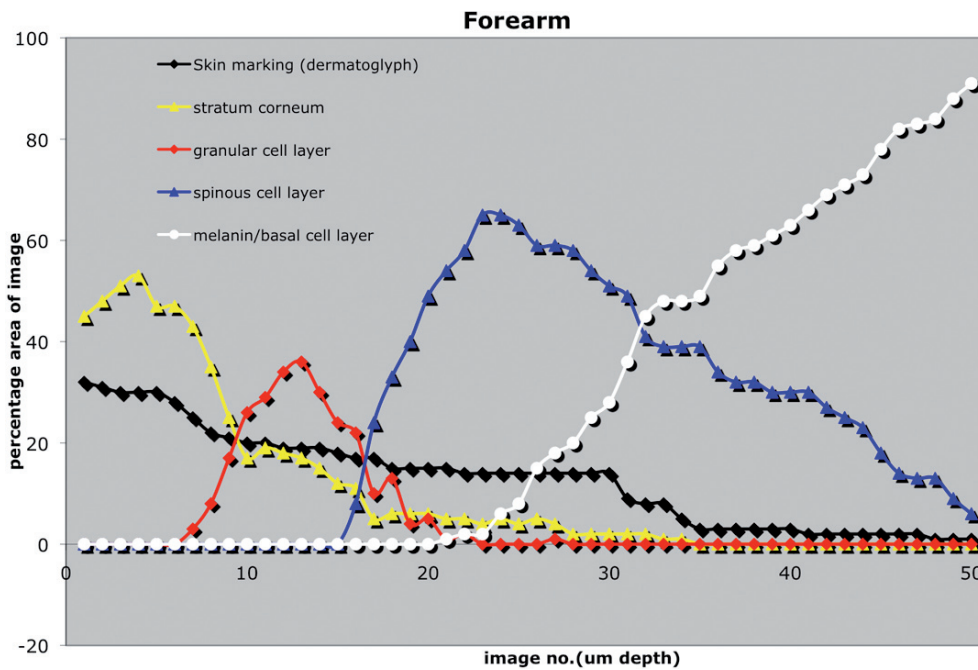
Fig. 2. The *percentage area* of each confocal image occupied by a particular epidermal layer (y-axis) from the skin surface (0 μm) to 50 μm depth (x-axis). (◆) represents a dermatoglyph, or skin marking, which appears as a dark "v"-shaped valley in the images that is widest at the skin surface and narrows with increasing depth. (▲) shows stratum corneum, which occupies the highest percentage at the skin surface but is still present at a depth of 27 μm due to folding of the epidermis. The first granular layer cells (◆) are seen at 7 μm and occupy most of the image at 13 μm, and are then overtaken by the spinous cell layer (▲) by 19 μm depth. At 16–17 μm a mixture of epidermal layers can be seen in the images, again highlighting the folded nature of the epidermis. The basal cell layer (○) is first reached at 22 μm by the appearance of "melanin caps" on the basal cells covering the dermal papillae.

percentage area occupied by a particular epidermal layer (including dermatoglyphs) for each confocal image at increasing depths (Fig. 2). Any individual square in which the contents were ambiguous or showed a transition, and therefore a mix of layers, e.g. SC to granular cell layer, were excluded from the graph (this accounts for the less than 100% total at any depth on the x-axis). At a depth of around 17 μm there is a "mixed picture" of epidermal layers, with SC accounting for approximately 5%, granular cell layer 10%, dermatoglyph 18% and spinous cell layer 26% of the image as a whole. This highlights the striking variability caused by undulations of the epidermal layers over a 500 μm square, as well as the difficulty involved in measuring thickness values from images on a horizontal plane, as they vary widely depending on which part of the image is measured.

DISCUSSION

This study examined the use of RCM for quantitative study of the epidermis and its components (3, 10). The results are discussed critically below in relation to other studies.

Two methods of determining SC thickness were examined. The first, the DM, offers the possibility of less operator bias and is based on the expected pattern of change in refractive index as the outer layers of skin are traversed. We know of no prior peer-reviewed publication of this method although the manufacturer has suggested that it is valid approach (personal communication, 2006). In our hands it is clear that optical artefacts vitiate this approach; results for the palm were clearly out of line with prior work and homogeneous inert materials showed the same artefact (discussed above).

The alternative approach is simply based on visual identification of the main epidermal and dermal structures and uses the depth of focus measure of the confocal microscope. Using this approach we looked at site variation and differences between people. We confirmed that three operators could arrive at similar measures for thickness, suggesting that the approach is robust. We found, as we expected based on previous work, site variation in SC, papillary length, and granular layer between different body sites, but not for epidermal thickness (palm was excluded and was obviously different) (14–16). Unlike previous workers we did not find differences between body sites that were more or less sun-exposed, except for papillary length; and in contrast to Huzaira (10) we found the papillary length to be less on the sun-exposed sites, although the difference was <20%. The grouping of sites into sun-exposed and sun-protected may be insensitive and conceal heterogeneity, as our location is not particularly sunny. For papillary length we were surprised to find that those with skin type I had smaller values than those of other skin types. The explanation for this is not known, and further work is required to confirm this result as the sample was very small. There is, however, a possible artefact that can affect measures comparing those with different skin types or skin colours. The low concentration of melanin in type I and II skin results in lower contrast in the confocal images (4) and a possible underestimation of papillae length in the absence of clear markers seems possible, as the top and bottom of the papillae are more difficult to identify than in darker skin types (4).

What was most striking in our results, however, was not that there was some variability *between* body sites; something we were expecting based on previous work

(1, 2), but that there was so much variation within a *particular* site. Those inured to conventional histology might find the degree of topographical variation unexpected: the degree to which normal unstressed skin is folded is significant and we cannot ascribe this variation to measurement artefacts.

A difficulty in interpreting our results, despite the fundamental nature of the topic, is that there have been few systematic studies with which to compare. Based on literature review we have summarized other findings, as shown in Fig. 3. The majority of studies have been small, particularly in terms of body sites studied, with perhaps the exception of the studies by Huzaira et al. (10) and Whitton & Everall (16). How do our results compare? Looking at epidermal thickness our data is in keeping with that of Whitton & Everall, but differences still exist, most noticeably for the upper front trunk and forehead. Some of these differences may be due to the well-known artefacts of histological processing or due to small sample sizes. Nonetheless, our absolute estimates are fairly consistent, showing little variation between sites, and the overall unweighted mean of our scores is 59.5 µm and those of Whitton 54.1 µm. These scores

are similar to those of Neerken (RCM) (17) and Corcuff (RCM) (9), but differ from those of Gamblicher (LM and OCT) (18), Sandby Moller et al. (LM) (15) and Batisse (HF Ultrasound) (19). For SC our data shows a broadly similar pattern between sites with a thickness of 5–10 µm. At the sites at which SC has been examined by previous workers, our results appear similar to those of Holbrook & Odland (LM) (20), Huzaira et al. (RCM) (10) and Sauermann et al. (RCM) (21). Other literature data, Neerken (RCM) (17) and Egawa et al. (CRS) (14) are very different. It is not possible to be dogmatic, but these comparisons suggest overall that the RCM, in our hands, gives broadly similar results to those that are considered the best of previous studies. However, we would emphasize that there is perhaps no gold standard, but that some techniques given their resolution (eg. OCT) would appear to have less face validity.

One significant disadvantage of RCM that has received little attention is that it can be very time-consuming. Because of the within-site variation, multiple sampling is required. In addition to this, we found that we could achieve better clarity of image by collecting confocal images manually, i.e. not using the automated z-axis
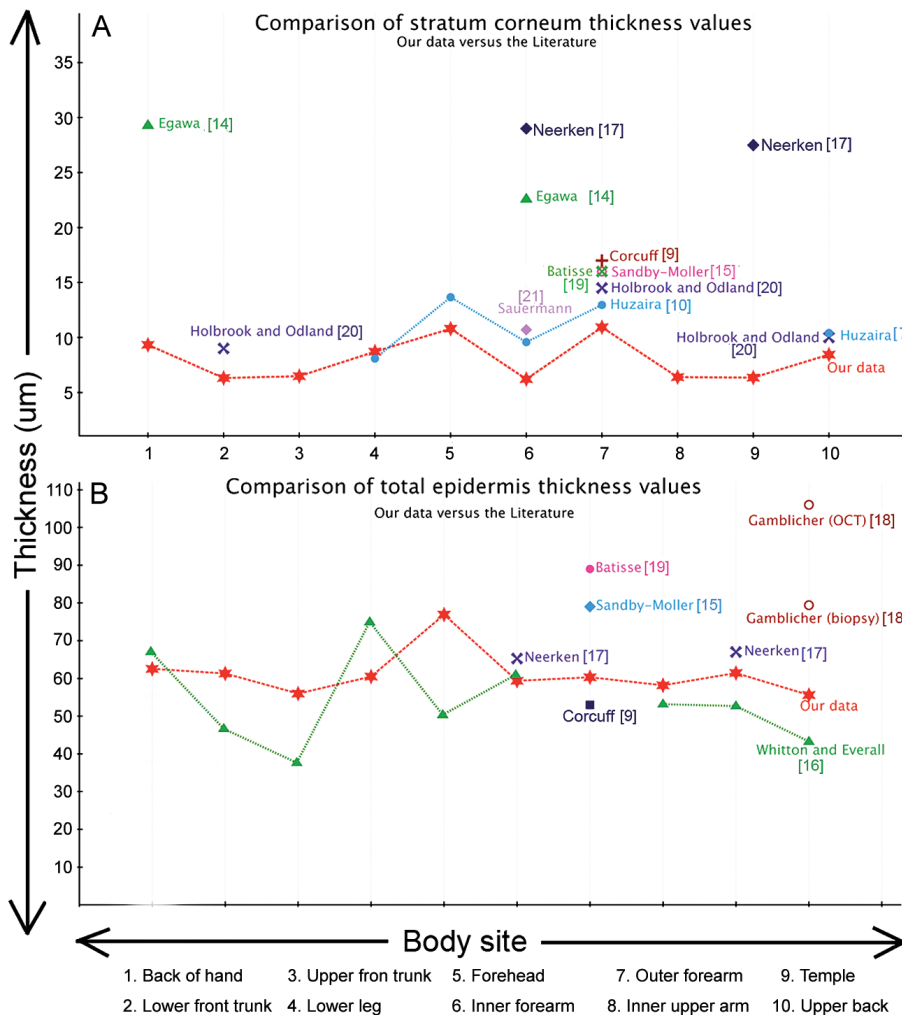

Fig. 3. Comparison of our thickness measurements with those from the literature. Mean thickness values from the literature are presented for: (A) stratum corneum and (B) total epidermis at 10 separate body sites. Our data is represented by --★-- on both graphs. Names and numbers refer to references cited in this paper.

stack collection function provided with the VivaScope. This was because we found it impossible to predict the pattern of increases in laser power necessary to achieve the optimal image clarity for each skin layer, skin type and body site. Even with the ability to stitch areas of examination into VivaBlocks with an area of up to 4 mm^2, recording each body site takes considerable time. For instance, if we assume (for illustration) that it takes 10 min to manually collect a 4 mm^3 stack (assuming a scan is performed every 5 μm from the skin surface to a depth of 100 μm), consisting of 64 individual z-axis stacks of 21 images, and approximately 5 min to measure epidermal layer thicknesses for each z-axis stack, then, it would take more than 5 h per body site per person. It is possible that software could be developed to allow the merging of multiple blocks, such that they could be manipulated and cell layer boundaries tagged more efficiently than is possible at present.

ACKNOWLEDGEMENT

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