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The distribution of melanin in skin determined *in vivo*

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Summary

Background There continues to be a need for objective, noninvasive methods to measure melanin concentration *in vivo* in human skin, independent of the confounding chromophore, haemoglobin. Existing methods are limited by a lack of specificity and inability to resolve the spatial distribution of these chromophores.
Objectives To validate and calibrate the measurement of eumelanin *in vivo* using SIAscopic™ techniques, relating this with histologically and analytically determined eumelanin concentrations in nonsun-exposed skin from subjects of Fitzpatrick skin types I–VI.
Methods Observations were made in five subjects from each of the Fitzpatrick skin types I–VI using chromophore mapping by contact and noncontact SIAscopy and other noninvasive spectrophotometric means. Measurements were performed on the inner aspect of both upper arms. Subsequently two 4 mm punch biopsies were taken from the inner upper arm, one per arm after injection of local anaesthesia. One biopsy was fixed in formalin and processed for histology; specifically, sections were stained for melanin using a silver staining technique and the amount of melanin was graded microscopically. The other biopsy was subjected to an analytical assay to yield precise quantitative measures of melanin. The correlation between the different methods of melanin measurement was determined.
Results Clear, significant correlations were obtained between contact and noncontact SIAscope-derived eumelanin values and actual eumelanin tissue content (determined both histologically and analytically), across the full range of Fitzpatrick skin types. There was no correlation between SIAscope-derived eumelanin and haemoglobin values, indicating efficient separation of the two chromophores.
Conclusions New contact and noncontact chromophore SIAscopic mapping techniques provide robust, rapid noninvasive measures of the concentration and spatial distribution of eumelanin *in vivo*, independent of haemoglobin, which correspond to true tissue values for this chromophore.

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Conflicts of interest
P.J.M. is employed by Procter & Gamble.

Human skin coloration is dependent almost exclusively on the concentration and spatial distribution of the chromophores melanin and haemoglobin, where melanin plays the dominant role in driving constitutive coloration.[1–4] Melanin is synthesized in membrane-bound organelles (melanosomes) within specialized cells (melanocytes) that supply these packets of pigment via dendritic processes to surrounding epidermal keratinocytes. Melanin is synthesized as two chemically distinct types, namely a brown or black pigment eumelanin and a yellow or red pigment phaeomelanin. Both these complex heteropolymers derive from dopaquinone (an oxidation product of tyrosine), although eumelanin consists of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid units, whereas phaeomelanin consists primarily of sulphur-rich benzothiazine derivatives. Of these two pigments, eumelanin is the dominant chromophore both in terms of epidermal fraction volume and perceived colour, phaeomelanin providing subtle nuances of skin hue.[1]

As eumelanin plays such a fundamental role, not only in skin appearance but also as the primary constitutive and inducible photoprotective molecule within human skin, there is considerable enduring interest in, and need for, methods to measure this chromophore *in vivo*. The Fitzpatrick skin typing system[5] is wrongly held by many to represent a subjective scale of pigmentation intensity – it was, of course, never intended as this and only reflects this attribute by inference. Other attempts to assess skin pigmentation subjectively by the human eye are almost always confounded by the presence of haemoglobin. In simple terms, while the human eye has superb contrast sensitivity[6] (down to only 2% of full

brightness) and can distinguish adjacent browns and reds with ease, it becomes virtually impossible to separate visually and/or communicate reliably the relative contributions of melanin and haemoglobin when they overlay one another, as is the case both in young or photoprotected skin (very homogeneous distribution of each chromophore) and older photodamaged skin (e.g. colocation of telangiectasia and lentigos or diffuse hyperpigmentation). Visual assessment, therefore, is at best semiquantitative and certainly not linear.

Objective approaches to determining skin colour *in vivo* centre around spectrophotometric or colorimetric approaches and the use of derived colour coordinates such as L*a*b*, and various digital imaging and image analysis techniques, reviewed in full by Pierard.[7] While these measures certainly bring objectivity to the measurement of skin colour, they still are not able to separate the individual contributions of the chromophores responsible for either the measured, integrated remittance spectrum or the final photographic image (no matter how high a quality it may be). Consequently, we have seen the development of the Melanin Index and Erythema Index (MI and EI, respectively), to try and provide a linear interval data scale for these chromophores.[4,7–11] Instruments that derive MI and EI [for example, the Mexameter™ (Courage & Khazaka GmbH, Cologne, Germany), the DermaSpectrometer™ (Cortex Technology, Hadsund, Denmark) and the Erythema/Melanin Meter™ (DiaStron Ltd, Andover, U.K.)] utilize the same basic approach, taking the log of ratios of reflectance within two or three selected wavebands in the visible and infrared. These approaches represent a significant step forward in the quantification of the chromophores responsible for skin colour, but are limited by (i) their limited measurement area (a maximum of approximately 10 mm diameter), (ii) their integration over the measured area, with no resolution of spatial distribution, (iii) their direct contact with the skin surface (which can lead to artefacts such as blanching through excessive applied probe pressure) and (iv) the inability of the log-ratio method completely to separate contributions from the two chromophores (see below).

A new measurement capability, SIAscopy (spectrophotometric intracutaneous analysis),[12–15] developed by Cotton and Claridge[12] and then Astron Clinica (Cambridge, U.K.), operates on the principle of chromophore mapping, that is, the *in vivo* measurement of the concentration and distribution of eumelanin, oxyhaemoglobin and dermal collagen, to produce mutually exclusive greyscale concentration maps of these chromophores. The SIAscope (Astron Clinica) is now a commercially available instrument and, while it has been shown to have excellent sensitivity and specificity in the early identification of malignant melanoma, the principle of chromophore mapping that it employs can be readily applied to normal, healthy skin.[12–15] The technique is based upon a unique combination of dermatoscopy, contact remittance spectrophotometry and hyperspectral imaging. In short, the SIAscope is able to obtain a high resolution composite white-light image of the skin over a defined area and provides four additional, mutually exclusive chromophore maps that display the concentration of epidermal melanin and haemoglobin, collagen and melanin in the papillary dermis, pixel by pixel. The dermal melanin endpoint is the key diagnostic criterion used in the diagnosis of melanoma, although this is not of concern with regards to normal skin.

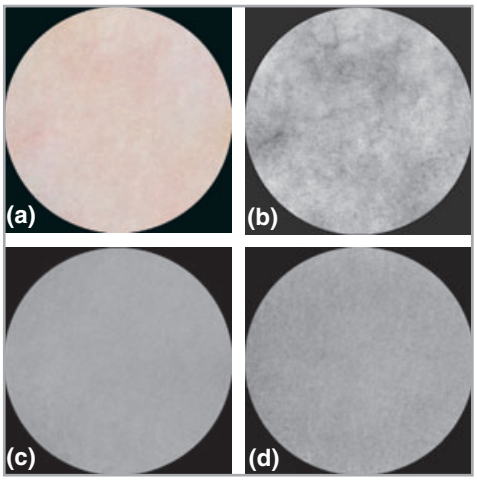

Fig 1. Example of SIAscope™ II chromophore maps taken from inner upper arm skin of subject 1 (type I skin; 12 mm diameter). (a) Composite white light image, (b) oxyhaemoglobin concentration map, (c) eumelanin concentration map, (d) collagen concentration map.

These images (corresponding to a 12 mm diameter circular field of view) now represent 8-bit 1024×1024 greyscale maps of chromophore concentration in PNG format image files. An example of these chromophore maps, obtained using a SIAscope II instrument, can be seen in Figure 1.

The contact SIAscope comprises a hand-held scanner with a flat glass-fronted probe, placed in contact with the skin using light, but firm, pressure (to avoid blanching). Further research by Astron Clinica has yielded noncontact SIAscopy™ (NCS) that overcomes the limitations of a skin contact probe. By necessity, this approach needs to be insensitive to local geometry and illumination intensity, in other words, the unavoidable artefacts of measuring 3D objects, rather than flat surfaces.

NCS is implemented[16] using an essentially conventional (although finely calibrated) digital camera and lighting system and may be used to acquire large-field eumelanin and oxyhaemoglobin chromophore maps. In deploying NCS, the camera is treated not so much as an imaging device, but more as a three-waveband spectrometer, making use of the RGB Bayer filter mosaic over the charge-coupled device (CCD). The spectral power distribution of the light source and the raw response of the CCD are determined accurately over the visible range (400–700 nm) and are supplied as calibration data to the NCS algorithms, based on the SIA mathematical model of light transport within skin. In short, for every pixel of the original RAW image, NCS calculations are performed to yield exclusive concentrations of eumelanin and oxyhaemoglobin. When recombined as an array, a parametric greyscale concentration map is produced, directly analogous to those calculated

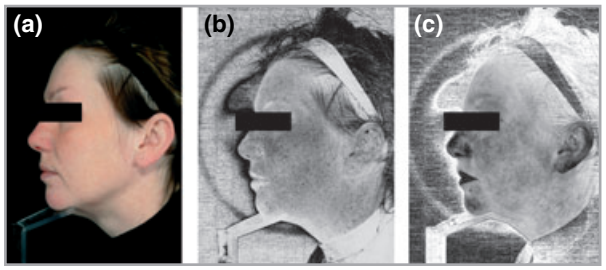

Fig 2. Example of full-face noncontact SIAscope™ chromophore maps (female subject aged 35 years). (a) Original cross-polarized white-light digital photograph, (b) eumelanin concentration map, (c) oxyhaemoglobin concentration map.

using the contact technique. It should be noted that a fully cross-polarized lighting system is needed, to eliminate specular reflection (that, by nature, contains no subsurface information). An example of the NCS technique applied to a whole face can be seen in Figure 2.

While both the contact and noncontact chromophore mapping approaches described above yield maps of eumelanin corresponding to a theoretical linear concentration scale, we wanted to validate and calibrate these approaches in comparison with other objective techniques, determined by histological and analytical means. The methods used and results from this work are described below.

Materials and methods

Ethical considerations

The study was designed to comply with the Guidelines for Medical Experiments in nonpatient human volunteers, initially published in the U.K. by the Association of the British Pharmaceutical Industry in March 1988 and further amended in May 1990. Ethical approval was obtained from the South East Wales Local Research Ethics Committee, Cardiff. The study complied with the current revision of the World Medical Association's Declaration of Helsinki (2000) concerning biomedical research involving human subjects.

Subjects

Thirty healthy nonpatient volunteer subjects aged 18 years or over who had given their witnessed, informed consent were recruited for the study. The study included 18 female and 12 male subjects. Subjects were recruited such that five subjects fulfilled one of each of the six categories given in the Fitzpatrick skin classification (Table 1),[5] yielding the total 30 subjects.

Measurement and biopsy sites

Measurements and biopsies were all taken from a 5 × 5 cm area delineated (by indelible marker) on skin within the centre of the inner aspect of each upper arm. This anatomical site was chosen because it should receive an extremely low lifetime dose of erythemal ultraviolet radiation (UVR), thus helping to ensure a minimum of chronic photodamage and, consequently, a homogeneous, normal distribution of eumelanin.

Table 1 Fitzpatrick skin type classification

Skin type	Appearance	Tanning ability
I	Very white or freckled	Always burn
II	White	Usually burn
III	White to olive	Sometimes burn
IV	Brown	Rarely burn
V	Dark brown	Very rarely burn
VI	Black	Never burn

Contact chromophore mapping

A SIAscope II commercial instrument was used to obtain contact eumelanin maps from within the measurement sites. A small drop of 20% (v/v) ethanol in water solution was placed on to the flat glass window at the front of the probe, before it was placed with gentle pressure (to avoid blanching) on to the skin in the centre of each 5 × 5 cm site (the ethanol in water acts as a matching fluid, eliminating optical aberration due to the refractive index of air). The acquisition time for the measurement was approximately 6 s, during which time the operator held the probe steady to avoid blurring of the final image set. Once the measurement was complete, the SIAscope II laptop computer calculated and displayed the respective chromophore maps, allowing immediate, real-time quality control over the images obtained. Each map represented a circular area of skin, 12 mm in diameter.

Eumelanin maps were saved as 8-bit 1024 × 1024 greyscale maps in PNG image file format, giving 256 possible concentrations of eumelanin and oxyhaemoglobin [where 0 (darkest greyscale) = highest chromophore concentration and 256 (lightest greyscale) = lowest chromophore concentration]. These concentration maps are, therefore, readily amenable to sophisticated image analysis techniques for calculation of a variety of relevant endpoints. In this case, custom algorithms written within image analysis software [Optimas™ 6.5 (Media Cybernetics LP, Silver Spring, MD, U.S.A.)] batch processed regions of interest that specified the entire captured field of the eumelanin map, to yield a mean greyscale value corresponding to the theoretical mean epidermal eumelanin concentration for that area.

Noncontact chromophore mapping

A Fuji S2 Pro 6·2 megapixel single lens reflex camera equipped with a Nikon AF Micro Nikkor 105 mm 1 : 2·8 lens was used to capture images of each inner upper arm, saved in both Fuji RAW and uncompressed TIFF format at a resolution of 3277 × 2226 pixels and 72 d.p.i. Lighting was provided by

a Sigma EF-500 DG Super flash source, operating in manual mode. Full cross-polarization was achieved by fixing a shaped polarizing sheet over both the camera lens and the flash aperture (orthogonally opposed to one another). All optical components within this imaging system, i.e. the CCD of the camera, the flash source and the polarizing filter had been previously calibrated by Astron Clinica to allow construction of noncontact eumelanin and oxyhaemoglobin chromophore concentration maps.

Custom SIA^{TM} algorithms batch processed the resulting RAW files to produce eumelanin and oxyhaemoglobin greyscale concentration maps analogous to those derived from contact chromophore mapping. Using image analysis software (Optimas^{TM} 6.5), a region of interest (ROI) was selected to encompass the entire 5 × 5 cm area in each chromophore map. Custom algorithms then batch processed these ROIs to yield mean greyscale values corresponding to theoretical mean epidermal eumelanin concentration for those areas.

Reflectance spectrophotometry

An unmodified commercial hand-held multiple angle reflectance spectrophotometer (X-Rite^{TM} MA68II, Elcometer Instruments Ltd, Manchester, U.K.) was used to obtain remittance spectra across the visible waveband (400–700 nm with a 10 nm spectral interval; the instrument was used with 10° standard observer and D_{65} illuminant) from within the centre of each 5 × 5 cm skin site (12 mm diameter circular sampling port). The instrument was calibrated before each measurement session using a dark-zero and white ceramic calibration standard. In use, it was placed on the skin surface with gentle pressure (to avoid blanching) and then held still during the 2 s acquisition time. Triplicate measures were obtained from each site. Data were exported as spectral percentage reflection values to a spreadsheet format for subsequent analysis.

Mexameter^{TM} measurements

An unmodified Mexameter^{TM} MX18 (Courage & Khazaka) commercial instrument was used to obtain a noninvasive index of skin melanin content (MI) and an erythema index (EI). The instrument calibration was checked before each measurement session to ensure it was within the manufacturer's limits. In use, it was placed gently on to the skin surface (to avoid blanching) and held still while the measurement was obtained (< 1 s; 5 mm diameter circular sampling port). Triplicate measures were obtained from each site and numerical MI and EI data recorded as hard copy for subsequent entry in to a spreadsheet format for analysis.

Skin biopsy

Skin samples were taken after the noninvasive measures had been performed. The skin was first anaesthetized using an intracutaneous injection of 1% lignocaine and a disposable 4 mm diameter trephine (Steifel Laboratories (UK) Ltd, High Wycombe, U.K.) within the centre of each 5 × 5 cm skin site. For each subject, one biopsy was placed immediately into 10% buffered formalin for subsequent histological processing and the other was placed into a sealed sample tube and placed immediately on dry ice for subsequent analytical work-up.

Melanin determination by microscopic examination of histological samples

The skin biopsies were fixed in 10% buffered formalin, dehydrated in graded alcohols and embedded in paraffin wax. They were then sectioned on a microtome at 5 μm and the sections stained (Von Kossa staining protocol). After mounting and drying, the sections were examined using an Olympus binocular microscope in a standardized manner using a 20 × objective lens. The sections were scored blind using a continuous visual analogue scale electronic meter (Vasmeter^{TM}, Innovaderm Recherches Inc., Montreal, QC, Canada) where a score of 0 signified no pigment and a score of 10 the heaviest pigment deposition possible. Examples of the degree of pigmentation observed are given in Figure 3. To obtain the scores, the microscope slide was placed with the coded label on the right side of the microscope stage and the first and third sections were assessed. Three adjoining fields in each section were scored and a mean value of the six fields used as the score for that specimen.

Melanin determination by analytical assay

Punch biopsies reserved for this analysis were first weighed, frozen (−70 °C) and then lyophilized, before transport to the laboratories of Professor Shosuke Ito and Dr Kazu Wakamatsu, Fujita Health University, School of Health Sciences, Toyoake, Aichi, Japan. Each biopsy was subjected to the microanalytical techniques developed by these researchers, based on the formation of pyrrole-2,3,5-tricarboxylic acid (PTCA) by permanganate oxidation of eumelanin and of 4-amino-3-hydroxyphenylalanine (4-AHP) by hydriodic acid reductive hydrolysis of phaeomelanin, respectively.[17] These specific degradation products were analysed quantitatively by high performance liquid chromatography (with UVR detection for PTCA and electrochemical detection for 4-AHP). For this, 1 ng PTCA approximated to 160 ng eumelanin and 1 ng 4-AHP approximated to 9 ng phaeomelanin. These assays, thus, yielded semiquantitative data expressed as ng mg^{-1} wet tissue of melanin type.

Statistical analysis

To analyse the relationship between mean greyscale data derived from both contact and noncontact melanin concentration maps and those from other endpoints, simple regression analyses were performed to yield the correlation coefficient r^2 and a *P* value for the slope of the correlation. The mean

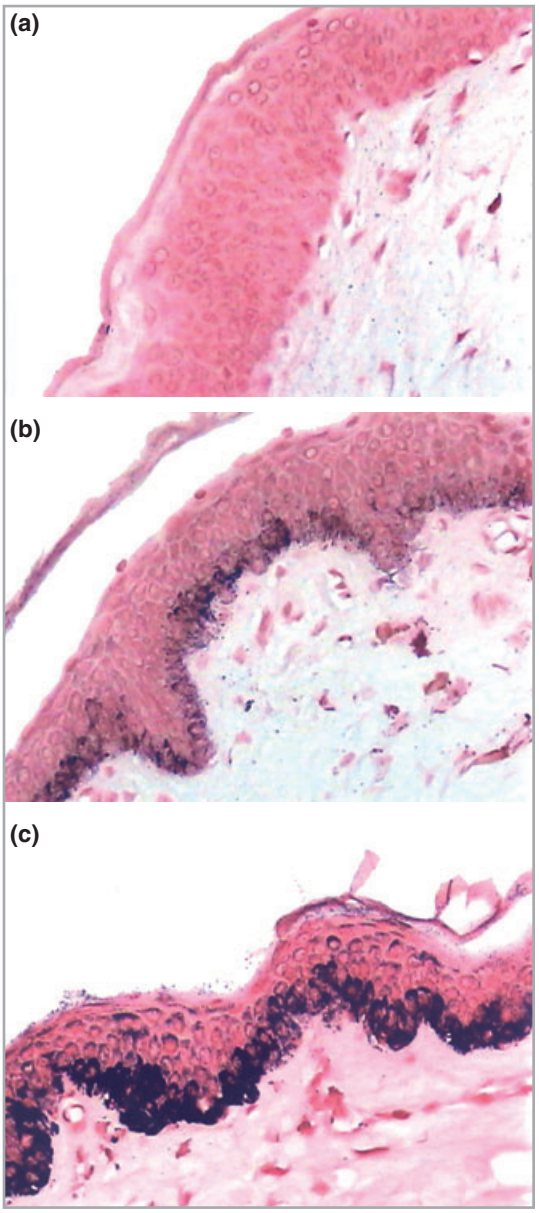

Fig 3. Examples of Von Kossa staining of epidermal melanin in different Fitzpatrick skin types (original magnification × 200). (a) Type I, (b) type IV, (c) type VI.

greyscale over the ROI in both contact and noncontact eumelanin maps was chosen as the summary metric because analysis of the distribution of greyscale values clearly showed a normal distribution of greyscale for all eumelanin maps (examination of greyscale frequency histograms for each map revealed a symmetrical distribution around both mean and median values, confirmed by associated skewness values). This confirmed the appropriateness of choice of a nonsun-exposed site such as the inner upper arm for this study. Simple regression analysis was chosen because, according to the first principles of the SIA method,[13,15] melanin and haemoglobin chromophore tissue concentration should, in theory, be linearly and inversely related to pixel greyscale (where darker pixels indicate higher chromophore concentrations for the purposes of intuitive map display). To compare all endpoints as a function of skin type, one-way ANOVA analyses were performed, using skin type as the main factor. The results of these analysis were then plotted as least significant difference means with associated 95% confidence intervals.

Results

Comparison of contact and noncontact SIAscope eumelanin greyscale

When mean greyscale values, corresponding to theoretical mean eumelanin concentration, obtained by both contact and noncontact techniques, were compared by simple regression analysis (Fig. 4a), an excellent linear correlation was observed ($r^2 = 88\%$; $P < 0{\cdot}001$). The regression line did not go through the origin, reflecting a small offset in greyscale between the differing camera systems (that can be taken account of in calibration vs. actual eumelanin values, reported later).

Comparison of contact and noncontact SIAscope eumelanin greyscale measurement with density of melanin staining in histological samples

When both contact and noncontact SIAscope mean greyscale values were compared with corresponding values for mean density of silver staining (Von Kossa protocol) in histological samples by simple regression analysis (Fig. 4b,c), clear correlations were obtained for each ($r^2 = 70\%$, $P < 0{\cdot}0001$ and 80%, $P < 0{\cdot}0001$, respectively).

Comparison of contact and noncontact SIAscope eumelanin greyscale measurement with absolute eumelanin and phaeomelanin tissue concentration

When both contact and noncontact SIAscope mean greyscale values were compared with corresponding values for mean eumelanin concentration (ng mg^{-1} wet tissue) by simple regression analysis (Fig. 4d,e), clear correlations were obtained for each ($r^2 = 84\%$, $P < 0{\cdot}0001$ and 77%, $P < 0{\cdot}0001$, respectively). In contrast, there was no correlation for both contact and noncontact SIAscope methods vs. phaeomelanin ($r^2 = 0{\cdot}1\%$, $P = 0{\cdot}89$ and $0{\cdot}3\%$, $P = 0{\cdot}79$, respectively).

Comparison of contact and noncontact SIAscope eumelanin greyscale measurement with Mexameter melanin index

When both contact and noncontact SIAscope mean greyscale values were compared with corresponding values for MI obtained using the Mexameter, clear correlations were obtained for each ($r^2 = 98\%$, $P < 0{\cdot}0001$ and 86%, $P < 0{\cdot}0001$, respectively).

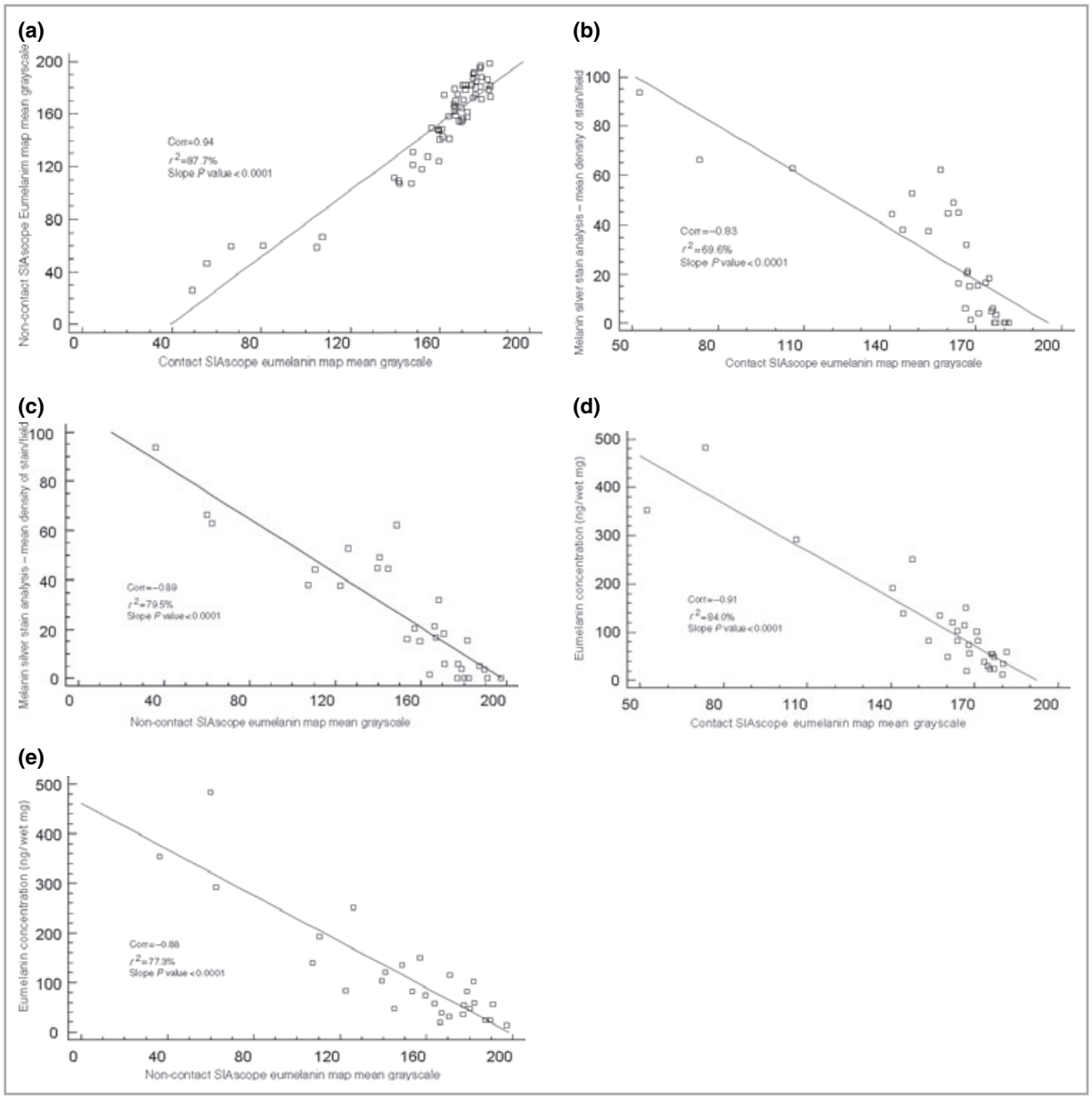

Fig 4. (a) Simple regression analysis of contact SIAscope eumelanin map mean greyscale vs. noncontact SIAscope eumelanin map mean greyscale, (b) simple regression analysis of contact SIAscope eumelanin map mean greyscale vs. mean silver stain density per field, (c) simple regression analysis of noncontact SIAscope eumelanin map mean greyscale vs. mean silver stain density per field, (d) simple regression analysis of contact SIAscope eumelanin map mean greyscale vs. analytically determined eumelanin tissue concentration (ng mg^{-1} wet tissue), (e) simple regression analysis of noncontact SIAscope eumelanin map mean greyscale vs. analytically determined eumelanin tissue concentration (ng mg^{-1} wet tissue).

Analysis of contact and noncontact SIAscope eumelanin and oxyhaemoglobin greyscale measurements, Mexameter-derived melanin and erythema indices, melanin density and absolute eumelanin tissue concentration by Fitzpatrick skin type

Comparison of the distribution of melanin values obtained from contact and noncontact SIAscope measurements, the Mexameter and the biopsy-derived melanin determinations by Fitzpatrick skin type yielded very similar trends (Fig. 5a–e). Melanin values over skin types I–IV increased incrementally, followed by a larger step increase to type V and the greatest increase to type VI.

Visual inspection of the distribution of both contact and noncontact SIAscope oxyhaemoglobin greyscale values by Fitzpatrick skin type (Fig. 5f,g) shows that there appears to be no relationship between the two parameters (corroborated by simple regression analysis comparing eumelanin and oxyhaemoglobin values derived from contact and noncontact SIAscope measurements, yielding weak correlations, $r^2 = 35\%$

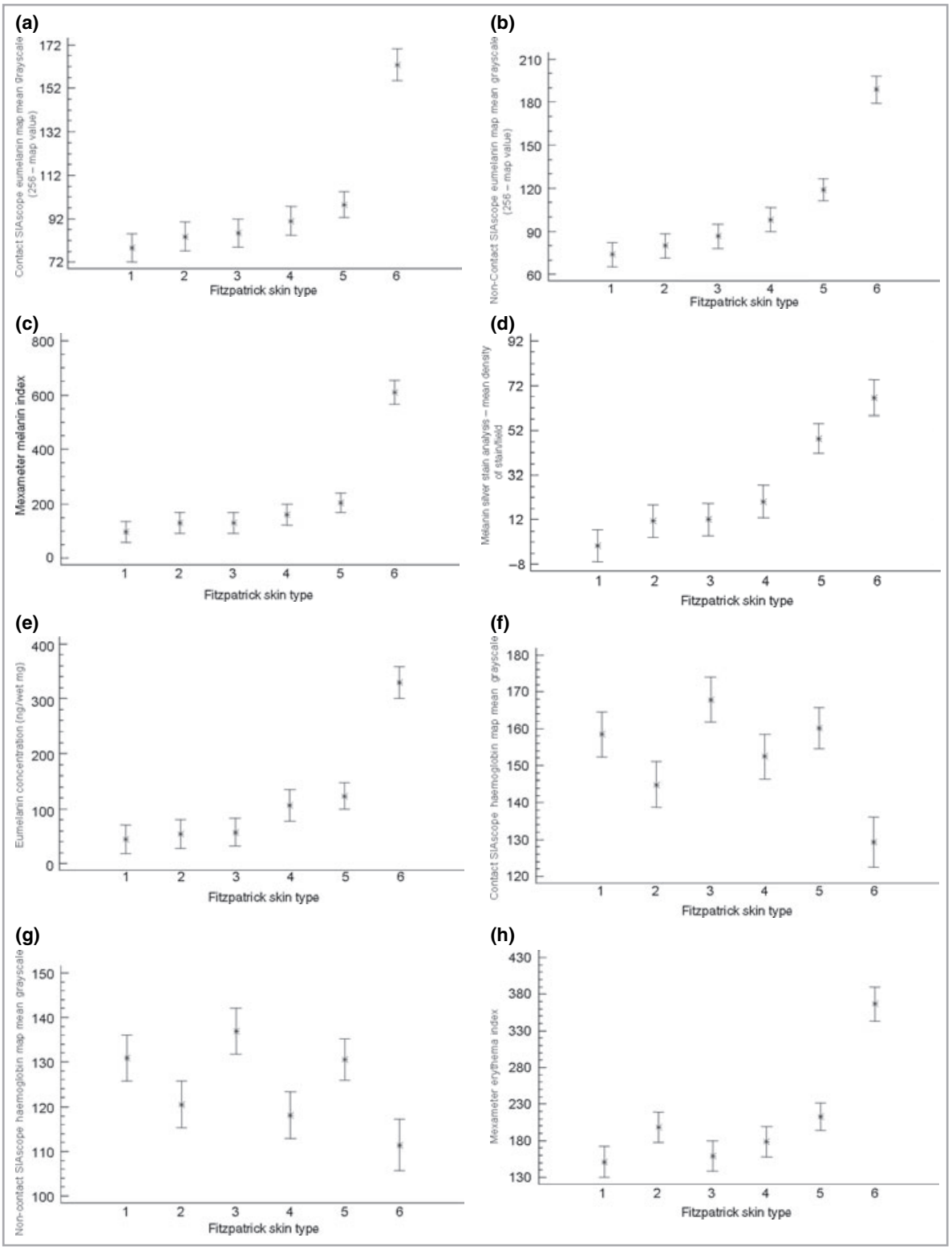

Fig 5. (a) Means and 95% least significant difference (LSD) intervals for contact SIAscope eumelanin map mean greyscale values (256 − map value) vs. Fitzpatrick skin type), (b) means and 95% LSD intervals for noncontact SIAscope eumelanin map mean greyscale values (256 − map value) vs. Fitzpatrick skin type), (c) means and 95% LSD intervals for Mexameter melanin index vs. Fitzpatrick skin type), (d) means and 95% LSD intervals for melanin silver stain density vs. Fitzpatrick skin type), (e) means and 95% LSD intervals for eumelanin concentration (ng mg^{-1} wet tissue) vs. Fitzpatrick skin type), (f) means and 95% LSD intervals for contact SIAscope haemoglobin map mean greyscale values vs. Fitzpatrick skin type, (g) means and 95% LSD intervals for noncontact SIAscope haemoglobin map mean greyscale values vs. Fitzpatrick skin type, (h) means and 95% LSD intervals for Mexameter erythema index vs. Fitzpatrick skin type.

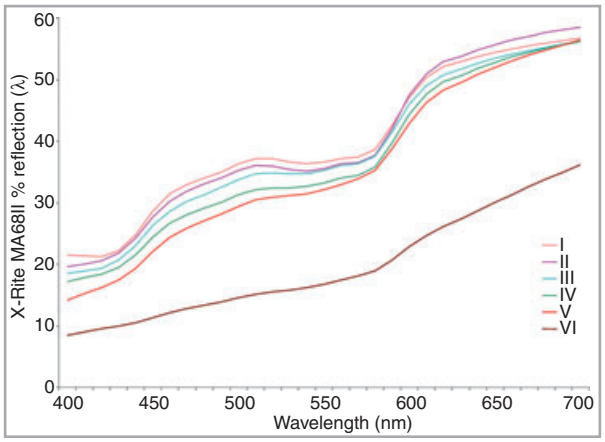

Fig 6. Spectral plot of percentage reflectance (400–700 nm) for Fitzpatrick skin types I–VI; data derived from X-Rite MA68II reflectance spectrophotometer (15° angle observer).

and 21%, respectively). In contrast, the Mexameter EI values yielded a distribution by Fitzpatrick skin type (Fig. 5h) very similar to that of the other melanin endpoints (and, indeed, if Mexameter melanin and erythema indices are compared by simple regression analysis, a clear correlation between the two is confirmed, $r^2 = 79\%$).

Reflectance spectrophotometric analysis of Fitzpatrick skin types

Figure 6 shows a spectral plot of percentage reflectance (400–700 nm) for Fitzpatrick skin types I–VI, using data derived from the X-Rite MA68II reflectance spectrophotometer (data taken from the 15° detector). The plot clearly shows the incremental decrease in reflectance across the entire 400–700 nm bandwidth with skin type, consistent with the steadily increasing epidermal fraction volume of melanin. As noted with the objective and analytical determinations of melanin above, the most dramatic change occurs between types V and VI. The plot also shows the characteristic flattening of the remittance spectrum of skin with increasing melanin concentration, with the gradual loss of the haemoglobin peaks seen in type I skin.

Discussion

The results demonstrate clear correlations between the eumelanin greyscale values produced by both the contact and noncontact SIAscope techniques and both histological and analytical values for melanin within the same specific area of human skin, across the whole range of Fitzpatrick skin types. It should also be noted that (i) there was no correlation of the SIAscope eumelanin values to analytically determined phaeomelanin tissue concentrations and (ii) likewise, no correlation between SIAscope eumelanin and oxyhaemoglobin values.

The same cannot be said for the other objective instrumental measure employed, the Mexameter, typical of approaches deriving MI and EI. While there was a relatively strong correlation between MI and SIAscope eumelanin values, there was also a clear relationship between Mexameter-derived melanin and erythema indices. No such relationship was observed for the SIAscope-derived eumelanin and oxyhaemoglobin endpoints. This phenomenon can be predicted from first principles. The Mexameter integrates reflectance within three specific wavebands with λ_{max} at 568 nm (relatively strong absorption for the melanins), 660 nm and 870 nm (relatively weak absorption for the haemoglobins). Equations 1 and 2 below show how the Mexameter MI and EI values are derived mathematically, where R denotes reflectance at the stated waveband:

Equation 1: $$MI = \log_{10}(R_{870nm}/R_{660nm}) \times 1000$$

Equation 2: $$EI = \log_{10}(R_{660nm}/R_{568nm}) \times 1000.$$

Visual inspection of the spectral plots of raw reflectance in Figure 6 and consideration of the algorithms above demonstrates how such a strong correlation can be possible between MI and EI in skin tissue of static blood status, but increasing pigment concentration. It can be seen that melanin has relatively greater absorption at 568 nm than at 660 nm. It could be predicted from Equation 2, therefore, that tissue with increasing melanin content but with static haemoglobin content (as is the case in this study) would give an increase in EI, proportional to MI. This is precisely what is observed in Figure 5h.

To summarize simply, the log-ratio method employed in a variety of commercial melanin and erythema meters is a very useful adjunct to skin colour measurement, in that it goes some way to measuring the chromophores responsible for the observed appearance. These methods are limited, however, by their inability to provide completely exclusive measurements of melanin and haemoglobin and, thus, the real risk of providing false positive readings where extremes of either chromophore coincide one with another (as is often the case in diseased or photodamaged skin).

The approach adopted by the SIAscope method is considerably more robust in that it employs algorithms incorporating a rigorous model of light transport, describing in full the possible intracutaneous interactions of light with human skin tissue. This unique approach allows inverse mapping, that is, from tissue colour to its precise histological values. The quantities of melanin, haemoglobin (and, for the contact approach, collagen) derived are then used to construct mutually exclusive parametric greyscale concentration maps. This array of pixels, therefore, representing the concentration of the chromophores responsible for human skin coloration, may be interrogated by sophisticated image analysis algorithms to yield quantitative information regarding the distribution of these entities. This is not possible with the point measurement employed by MI or EI instruments. The importance of spatially resolved chromophore measurements cannot be

over-emphasized as they now provide the investigator with a means to understand and explain, on a molecular basis, non-homogeneities in the colour appearance of the skin tissue in question.

Importantly, this noninvasive method for eumelanin determination is potentially useful as a new clinical tool, for example in the measurement, quantification and tracking of photodamage endpoints (e.g. lentigos and diffuse hyperpigmentation), skin disease states (e.g. pigmentation disorders such as vitiligo) and induced melanogenesis (e.g. UVR therapy, delayed pigment darkening endpoints), independent of haemoglobin-based features.

In summary, these data provide confidence that both contact and noncontact SIAscope chromophore mapping techniques provide robust and rapid noninvasive measures of the concentration and spatial distribution of eumelanin *in vivo*, which correspond to true tissue values for this chromophore.

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