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Melanin fate in the human epidermis: a re-assessment of how best to detect and analyze histologically

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Abstract

Melanin is the predominant pigment responsible for skin colour, and is synthesized by the melanocyte in the basal layer of the epidermis and then transferred to surrounding keratinocytes. Despite its optical properties, melanin is barely detectable in unstained sections of human skin. However, identification and localization of melanin is of importance for the study of skin pigmentation in health and disease. Current methods for the histologic quantification of melanin are suboptimal, and are associated with significant risk of misinterpretation. The aim of this study was to re-assess the existing literature, and to develop a more effective histological method of melanin quantification in human skin.

Moreover, we confirm that Warthin-Starry (WS) stain provides a much more sensitive and more specific melanin detection method than the common-place Fontana-Masson (FM) stain. For example, WS staining sensitivity allowed the visualization of melanin even in very pale Caucasian skin that was missed by FM or Von Kossa (VK) stains. From our re-assessment of the histology-related literature we conclude that so-called 'melanin dust' is most likely an artefact of discoloration due to non-specific silver deposition in the stratum corneum. Unlike FM and VK, WS was not associated with this non-specific *stratum corneum* darkening, misinterpreted previously as 'degraded' or so-called 'dust' melanin. Finally, WS melanin particle counts were largely similar to manual counts by transmission electron microscopy, in contrast to both FM and VK. Together these findings allow us to propose a new histology/Image J-informed method for the accurate and precise quantification of epidermal melanin in skin.

Introduction

Melanin is the predominant pigment responsible for the perception of human skin colour. In this tissue, the melanin indole biopolymer is synthetized *via* the Raper-Mason biochemical pathway within the melanosome (1), a unique lysosome-related organelle of the melanocyte – a cell that is located in the basal layer of the epidermis. One melanocyte (MC) partners with up to about 36 viable keratinocytes (KC) to form the functional epidermal-melanin unit (2). Amongst other

important interactions, this unit engages in the transfer of melanin granules from the MC to the KC. This interaction is biologically unusual, *i.e.* it is rather exceptional that an organelle produced by one cell type is transferred to a different histological cell type. Once mature melanosomes distribute intracellularly to the periphery of the MC, they are gradually transferred to, and accumulate in, neighboring KCs. The exact mechanism(s) of melanin transfer has remained controversial; several hypotheses proposed. These have included : (i) cytophagocytosis of melanin-containing MC dendrite tips (3,4) (ii) exocytosis of melanosomes and subsequent uptake via phagocytosis into KC (5,6) (iii) shedding of melanosome-rich cytoplasmic packages by MC which subsequently are phagocytosed by the KC (7–9). However, we and others have proposed an alternative, though not mutually exclusive mechanism involving (iv) filopodia-mediated melanosome transfer, which may drive inter-KC distribution of MC-transferred melanin (10–14). In the epidermal KC, melanin is transferred to form UV-protective supra-nuclear caps.

Given that skin pigmentation is a critical phenotypic adaptation for UVR-drenched terrestrial life, and is also a powerful social signal, there is much interest in the demonstration, localization and quantification of melanin in the epidermis in health and disease. Although the role of melanin synthesis, melanosome biogenesis and melanin granule transfer in melanomagenesis is unclear, its absence in amelanotic melanoma can effect early detection and so a timely diagnosis and intervention with serious consequences.

There are several hyper- or hypo-pigmentary conditions that present within healthcare-associated dermatology or more personal-care contexts (*e.g.* age-related changes to skin pigmentation). These can have significant psychologic morbidity. Thus, understanding the regulation of melanin flow in the human epidermis is a basic science challenge with worldwide clinical and medical and consumer relevance. Despite this, we lack reliable, sensitive and specific histologic methods to quantify and assess melanin distribution in skin. Therefore, the current study was designed to reassess the literature in this area, and aimed to devise more effective methodologies for detecting and tracking melanin fate in the human skin epidermis.

The production and maturation of melanosomes (as a proxy for melanogenesis levels) can be readily surveyed by detecting their origin within MC by immunohistochemistry (IHC) using highly specific antibodies against melanogenesis- and melanosome-specific proteins. Examples of common immuno-detectable melanosome antigens include premelanosome protein or PMEL (HGNC:10880; also known as PMEL17/SILV/gp100), tyrosinase or TYR (HGNC:12442), tyrosinase-related protein 1 or TYRP1 (HGNC:12450), and to some extent dopachrome tautomerase or DCT (HGNC:2709; also known as tyrosinase-related protein 2 or TYRP2). Expression of these proteins and melanogenesis-associated enzymes is readily detected inside the MC melanosome

where melanin is synthesized. However, their antigenicity is mostly lost when melanin granules become incorporated into KC-derived phagosomes (so-called phagomelanosome) (6). Alternatively, melanogenesis can be detected histochemically *via* the detection of dopa-oxidase activity of tyrosinase. However, no antibodies exist to detect the melanin polymer itself and so the fate of the melanin granules during KC differentiation and stratification of the epidermis can only readily be analyzed *in situ* by transmission election microscopy (TEM).

An historical perspective on the development of histologic stains for the detection of melanin in skin

For decades the detection of melanin in tissues has relied upon proxy staining methods, as is described briefly below.

Fontana-Masson (FM) stain: The FM stain has become the 'gold-standard' for demonstrating melanin granules in both formalin-fixed paraffin embedded (FFPE) and frozen tissues. Although now widely used for detecting melanin in skin, this stain was originally designed to detect spirochetes (*Treponema pallidum*) in tissues by Arturo Fontana (1873-1950) in 1912 (15), used by Pierre Masson (1880-1959) to demonstrate the enterochromaffin cells (16). The FM stain was attractive to cutaneous researchers because of the capacity of melanin to reduce diamminesilver(I) nitrate(V) to an intensely black precipitate on this tissue, which was easily observable under bright-field light microscopy.

Less than 10 years after the publication of the FM stain Aldred Scott Warthin (1866-1931) and Allen Chronister Starry (1890-1973) developed another spirochete-staining procedure; the so-called Warthin-Starry (WS) stain (17).

Warthin-Starry (WS) stain: The WS stain is also based on silver nitrate but with (originally) the addition of pyrogallol (now replaced by hydroquinone) (17). Although microbiologists rapidly adopted and developed the WS stain, it has largely been ignored by skin researchers and dermatologists. A further enhancement of the WS stain was made about 35 years ago when Warkel and colleagues showed that its reaction with melanin in skin tissue was improved by decreasing the pH of reaction solution from pH 4 to 3.2 (18). Importantly, the WS stain can highlight immature (stage III) melanosomes (seen by TEM) in amelanotic melanoma (19) than can the much-less-specific FM stain. In fact, with TEM assessment FM stain can be seen to react also with other cellular structures, even nuclei **(22)**. Alas, other than a sole article published in 1993 (20), the WS stain has been largely lost from cutaneous sciences research practice for the evaluation of melanin in human skin.

Von Kossa stain: The Von Kossa (VK) stain is a widely-used stain to detect evidence of tissue mineralization especially calcium. It is based on silver nitrate reduction by melanin. Recently, the VK stain was used for the demonstration of melanin in human skin (21), but yielded a relatively weak signal and has not gained any significant traction for this purpose in the general cutaneous biology literature.

Please see Supplementary Data for a brief description of the components of each stain, and their advantages and disadvantages for skin melanin detection.

Histochemical versus immunohistochemical detection of melanin in human skin

Immunohistochemistry (IHC) is a very sensitive method of detecting MC-melanosome specific antigens in human skin. However, it is also a most labile method, due to its obligate dependence of optimal preservation of antigenicity, which can be easily reduced or lost depending on fixative type, duration of fixation and epitope masking in the case of FFPE. Moreover, the preferred cryo-section route is complicated, despite its association with native (un-cross-linked) antigens, by the relatively short antigen 'shelf-life' and low histological preservation/ resolution. By contrast, the melanin indole polymer itself is extremely resistant to degradation, and silver nitrate staining can be performed on even poorly-preserved tissues to reveal without difficulty melanin in both FFPE and frozen sections. For example, we have been able to demonstrate melanin in FFPE sections from skin maintained in 10% formalin at room temperature for more than 15 years (Figure 1a). Although the tissue was unsuitable for immunolabelling (due to very high levels of background autofluorescence), we could demonstrate classically-described supra-nuclear melanin capping in basal KC, and with melanin distribution across the entire epidermis that was consistent with results obtained with normally-processed fresh FFPE or frozen specimens.

A reassessment of the interpretation of so-called 'melanin dust' in the stratum corneum of the human skin

One of the most cited, but most poorly-evidenced, aspects of epidermal melanization is so-called 'melanin dust', long purported to be present in the *stratum corneum*. This 'dogma' of skin pigmentation science can be prominently found in skin (pigmentation) reference books, where the term is used to refer to degraded particulate melanin granules that have reached, *via* epidermal stratification, the suprabasal layers of the epidermis, most particularly the *stratum corneum*.

We have always found this reference of 'melanin-dust' puzzling. Melanin dust has been used to

explain the FM-associated reaction of ammoniacal silver nitrate in the corneocyte *i.e.* the diffuse black staining associated with the stratum corneum. However, melanin itself has never actually been observed in a particulate or 'dust' form in this layer of the epidermis even using ultrastructural analysis via transmission electron microscopy (TEM) (22) or even after FM staining of squames removed by tape-stripping (23).

Remarkably, from our extensive bibliographic search, it has been impossible to find any experimental evidence for the existence of so-called 'melanin dust' in the *stratum corneum* of Caucasians. No primary literature exists, and the only detailed reference we could find was a description from 1963 in a review article (24) stating that vitiligous and albino skin do not display this 'melanin dust' after FM staining. However, to paraphrase Sagan, evidence of melanin-dust absence in vitiligo/albino skin cannot be used as evidence of its presence in normal skin.

With this now clearer understanding of the risks of staining artefacts impacting on our knowledge of skin pigmentation, and in order to identify a more robust procedure to better demonstrate and quantify melanin within human epidermis, we conducted a comparative analysis of FM, WS, and VK stains on very thin (*i.e.* 3-µm) cryo-sections (for optimal resolution of melanin granules at relatively low magnifications) of normal human sun-protected skin from male and female donors of varied skin phototypes, as well as on mouse pelage skin epidermis that lack MC and so with no possible source of melanin granules in this skin compartment. We developed a simple method to quantitatively analyze the results obtained, and to compare these with a manual count of melanin granules transferred to KC by TEM (22) using ImageJ software (1.48v; a public domain, Java-based image processing program developed at the US NIH).

Warthin-Starry stain: the preferred staining method for melanin detection in human skin epidermis

In human and mouse skin FM stain imparts a uniform yellow coloration to the *stratum corneum* after approximately 10 minutes incubation, which darkens further to deep brown after 2 hours incubation (Figure 2a-f) due to the deposition of metallic silver. FM stain also contains a gold-chloride 'toning' step that converts the deep brown stain to intense black. This occurs as 3 silver atoms are substituted by 1 more photo-dense atom of gold, which helps 'clear' some background discoloration. However, a major drawback of the FM stain, particularly with toning, is that is can fail to detect the relatively low melanin amounts in some Caucasian donors (*i.e.*, did not in all cases detect melanin in our pale skin phototype (SPT) I samples), and can fail to accurately reveal the correct distribution of melanin especially in different strata of the epidermis.

Furthermore, VK staining imparts a deep brown background over the entire epidermis and this can obscure the easy identification of melanin granules, especially in some compartments of the epidermis.

By contrast, a 2-minute incubation with WS stain can reveal a substantial number of deep black silver colloids (Figure 2g) even in very pale skin of phototype I, most predominantly located in the *stratum basale* compared with the suprabasal layers. Importantly, unlike the FM stain, the melanin signal revealed by WS stain is apparent before any background discoloration is seen. Background discoloration (including yellowing of the suprabasal layers) emerges with prolongation of staining even with WS (Figure 1c).

During the current study we found that the WS stain may also be useful for the study of melanin transfer in the upper hair follicle (infundibulum), where WS revealed a complete absence of melanin from the suprabasal layers (Figure 1b), despite the obvious presence of dopa oxidase-positive and melanogenically-active melanocytes in this region. Only the WS stain was able to reveal a clear contrast in staining between the deeply-stained melanin granules of the inter-follicular epidermis basal layer and the strikingly clear hair follicle infundibulum. **This phenomenon was observed in all the haired skin tissues investigated, from different phototypes and different body location (i.e. arm, foreskin and abdomen).**

Importantly, the WS stain can also be performed with a silver nitrate impregnation step. In this configuration, the reaction will reveal, in addition to melanin, a multi-color contrasted background: *i.e.*, with orange cytoplasm, yellow intercellular space and stratum corneum, and with striations corresponding to the tight junctions. The cell nucleus is also easily recognized with its orange-red nucleolus and deep-red nucleolus organizer regions (Figure 1a). The dermis appears pale yellow with recognizable fibers and nerves.

A quantitative analysis of melanin in human skin epidermis using ImageJ freeware

After first describing how melanin can be revealed and highlighted in different epidermal compartments by the various stains used here, we also aimed to quantify melanin distribution in situ (please see the protocols described in the Supplementary data). For this part of the study, we stained frozen skin sections of 20-30 year old skin male and female phototype I-VI donors (n = 12, 6 sections analyzed by donor) collected from photo-protected areas with either WS, FM or VK stains, and compared the amount and distribution of the stained melanin granules with data obtained by direct counting of melanin granules with TEM **(22)**. Only WS stain revealed melanin counts that

closely approximated direct counts by TEM. Both TEM and WS methods revealed that the concentration of melanin (*i.e.* melanin granules per unit surface area) decreases by approximately 80% upon transitioning from the stratum basale to the first layer of stratum spinosum (Supplementary Figure). By contrast, both the FM stain and VK stain dramatically underestimated the concentration of melanin granules in the suprabasal layers of the epidermis, *i.e.* both stain revealed only approximately 5% of melanin load in the basal layer as distributed in the first suprabasal layer and more distally.

In summary, we propose that WS stain, with or without image analysis, be adopted as the preferred methods to reliably quantify melanin distribution in human skin and hair follicle. Complementary analyses would be beneficial to further evidence the superiority of the WS stain, i.e. microdissection of the different layers of the epidermis followed by melanin quantification with high-performance liquid chromatography (HPLC), at least to appreciate the potential differential behaviour of eumelanin and pheomelanin with this stainning procedure. We are now in the process of using this and other methodologies to better understand the behavior of the epidermal- and follicular-melanin units in health and disease states.

Acknowledgements

All the authors designed the research project. NJT performed the research. NJT and DJT analysed the data and wrote the manuscript. JIDW and MB provided feedback on the early drafts and also provided the human skin samples.

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Conflict of interests

None.

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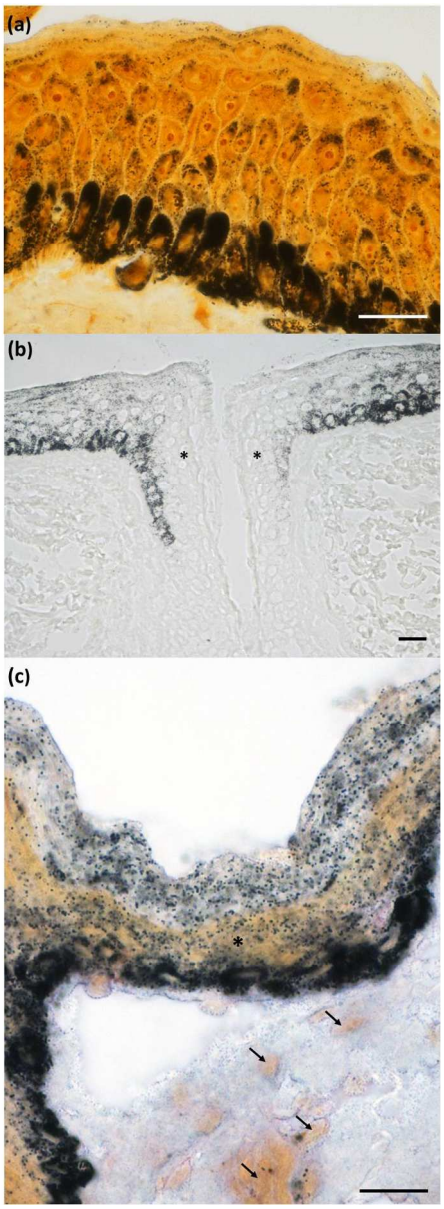
Figure Legends

Figure 1 – Warthin-Starry stain. (a) The staining procedure performed in 15 seconds with a

preceding silver nitrate impregnation on a phototype IV skin. Melanin appears in black. The impregnation step gives the several shades of yellow-orange that highlight structures in the epidermis (nucleolus, nucleus, cytoplasm, tight junctions) and in the dermis. (b) The same procedure without the silver nitrate impregnation step. Only melanin is stained without any background. The suprabasal layers of the infundibulum hair follicle appear melanin-free (black asteriks). (c) WS stain for 4 minutes. Other components of the tissue start to react to silver nitrate in the epidermis (black asterik) and in the dermis (black arows), creating a background/counterstain-like on the slide. Bar: 20 μm.

Figure 2 – Appearance of melanin 'dust' artifact with the FM stain (a-f), but not with the WS stain (g). Skin sections from a pale skin phototype I is stained for 10 minutes (a and b), 30 minutes (c and d) or 2 hours (e and f) with the FM stain, then treated with (a, c and e) or without (b, d and f) gold chloride. From 10 minutes, we can observe the uniform darkening of the *stratum corneum,* even if melanin granules are missing (black asteriks). Silver depositions, unlikely to characterize melanin, can be seen (black arrows). Note that the toning step removes sparse silver colloids, providing a FM stain-negative result. (g) WS stain performed for the same sample in 2 minutes. Even overstained, the tissue does not exhibit much darkening of the *stratum corneum* whereas melanin granules are obviously stained. Dotted lines represent the dermis-epidermis junction. Bar: 20 μm



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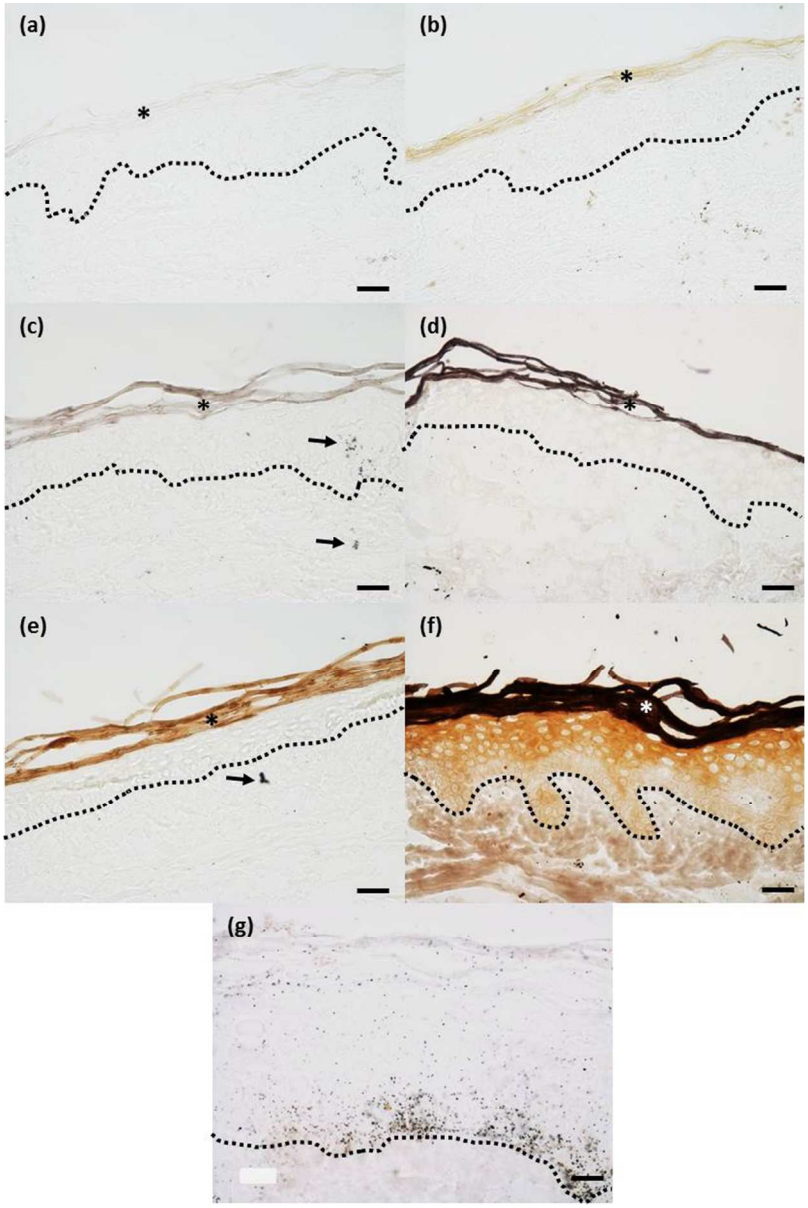
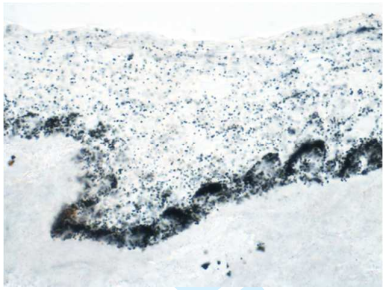


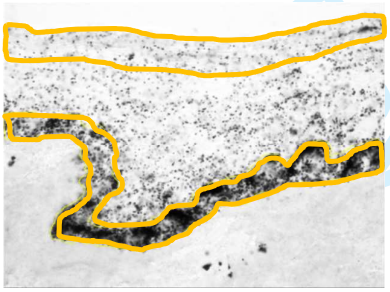
140x210mm (150 x 150 DPI)

Supplementary Protocol

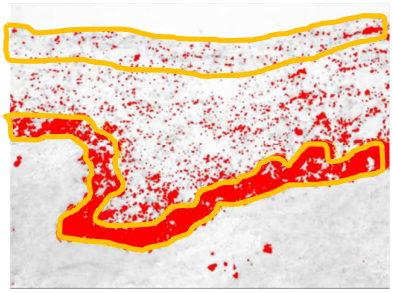
Analysis of WS-stained skin sections with ImageJ



Pictures are opened in ImageJ. They are captured in RGB from bright field view under the microscope.



After **grayscale transformation**, the epidermis is divided in 3 segments: the stratum basale (SB), the stratum spinosum/stratum granulosum (SSSG) and the stratum corneum (SC) *via* the ROI Manager (here in orange).



With the **Threshold Manager**, we identify the silver deposition by varying the level of grey to the appropriate unit (here in red). The **fraction of area occupied by melanin** and the **mean shade of gray of the melanin area** (between 0 and 255) is recorded for the 3 segments. The product of both parameters represents the **integrated darkness of the melanin area**.

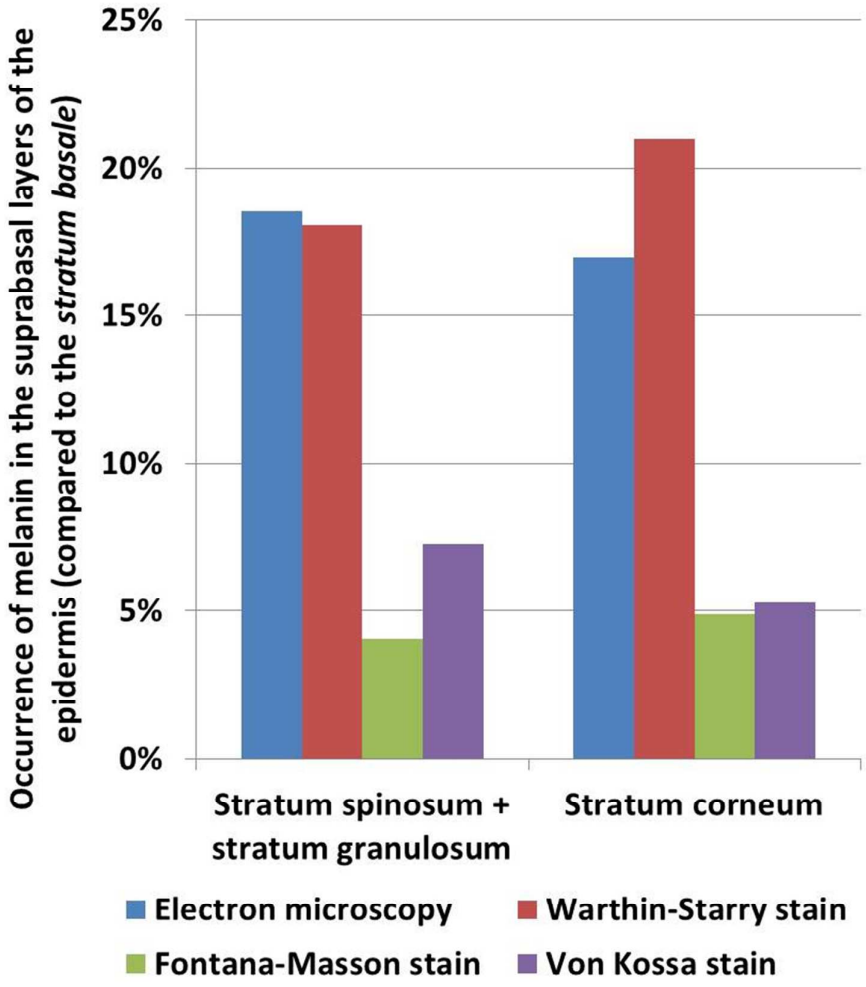
For skins phototype I-IV and hypopigmentation conditions, the use of the area occupied by melanin is sufficient to quantify the melanin distribution across the epidermis. For higher skin phototypes *i.e.*, V-VI and hyperpigmentation conditions, the silver staining may saturate the *stratum basale*: integration of the level of gray fixes this issue.

Supplementary Table

Comparison of the melanin staining procedures based on silver nitrate

	Fontana-Masson (FM) stain	Warthin-Starry (WS) stain	Von Kossa (VK) stain
Reactive chemicals	Ammoniacal silver nitrate (2.5%)	• Silver nitrate, pH 3.2 (0.4%) • Hydroquinone, pH 3.2 (0.4%)	Silver nitrate (5%)
Duration	30-60 minutes at 60°C (1-2 minutes in the microwave)	15 seconds to 2 minutes at 54°C *Facultative pre-impregnation in silver nitrate, pH 3.2 (1%) for 30 minutes at 43°C*	30-60 minutes at 60°C
Toning with gold chloride	Advised for toning the signal and decreasing the background but will dim the signal too	Useless	Advised for toning the signal and decreasing the background but will dim the signal too
Issues	Darkening of the *stratum corneum*	/	Strong grey background
Sensitivity and specificity	*	***	*

Supplementary Figure



Evaluation of the melanin persistence across the human epidermis. By comparison between manual phagomelanosomes count in KC by transmission electron microscopy**, which was performed in ref.22,** and by FM, WS, and VK histological procedures for melanin staining**, performed in this study**, only the WS stain provides a result similar to a manual TEM counting (TEM: data mined (ref.22); others: n = 12).