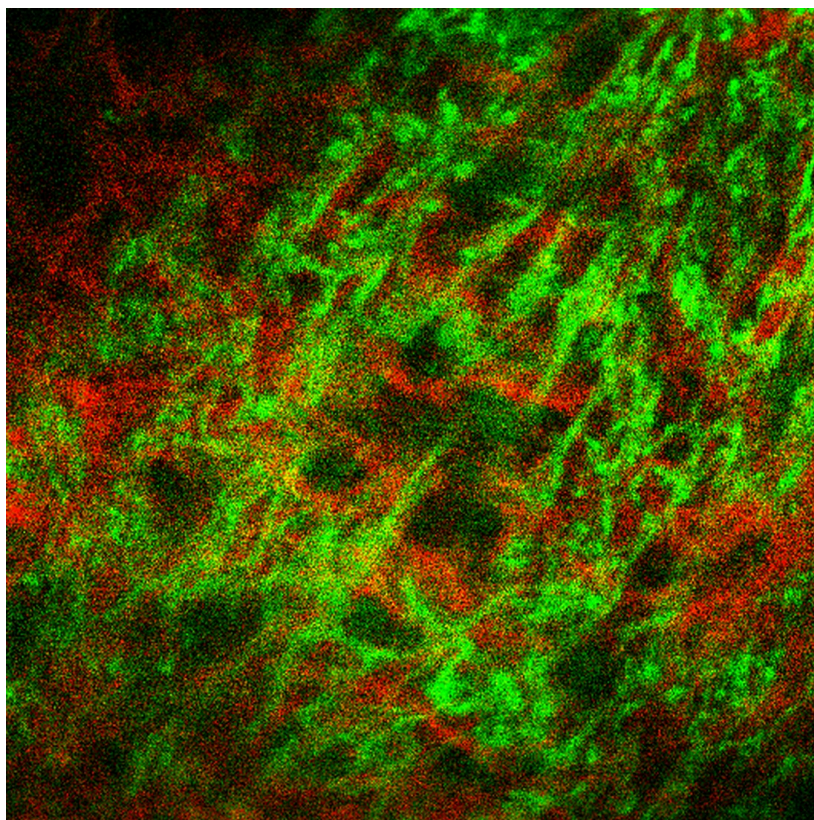


Atlas of *Normal Skin* Images on Two-Photon Microscopy

FIVE LAYERS · THREE ANATOMIC SITES · 17 SUBJECTS, 23–68 YEARS



COVER Dermis of the left cheek at $\sim 84\ \mu\text{m}$, 29 years, Fitzpatrick II. Fibrillar collagen in the harmonic channel, elastic fibres in the fluorescence channel. Field $140 \times 140\ \mu\text{m}$.

A gallery of label-free two-photon images of clinically normal human skin, from the surface of the stratum corneum to the dermal matrix. Two figures for each of five layers: the features that identify it, then the range it takes across phototype, anatomic site and age.

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Atlas of Normal Skin Images on Two-Photon Microscopy

Fluorescence and Harmonic Images of Clinically Normal Skin

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IMPORTANCE

In vivo two-photon microscopy resolves the layers of living skin at sub-cellular detail without stains or excision, but published normal-skin references are drawn from small, narrowly sampled series, and few show the same layers across several anatomic sites in the same person.

OBJECTIVE

To assemble a reference gallery of label-free two-photon images of clinically normal human skin spanning five compartments — stratum corneum, stratum granulosum, stratum spinosum, the dermal–epidermal junction and the dermis — at three anatomic sites differing in chronic sun exposure, and to record the depth at which each compartment is reached.

DESIGN, SETTING AND PARTICIPANTS

Imaging series of 17 adult volunteers with clinically normal skin, aged 23 to 68 years, Fitzpatrick types I to VI, each imaged at the left cheek, left forearm and back with a handheld two-photon system. Stacks were acquired from the stratum corneum surface to 120 μm at 2.00 μm per optical section. For every layer, the clearest eligible optical sections in the series were selected and arranged to isolate phototype, anatomic site and age in turn.

MAIN OUTCOMES

Five layers are presented in two figures each: a recognition page carrying one reference section beside the five features that identify the layer and the error most easily made at that depth, and a variation page in which phototype, anatomic site and age are varied one at a time. Median depth was $\sim 4\ \mu\text{m}$ for the stratum corneum, $\sim 16\ \mu\text{m}$ for the granular layer, $\sim 24\ \mu\text{m}$ for the spinous layer, $\sim 43\ \mu\text{m}$ for the junction, and $\sim 78\ \mu\text{m}$ for the dermal sections; ranges overlap between adjacent layers and are tabulated.

KEYWORDS

Two-photon microscopy · second harmonic generation · in vivo imaging · normal skin · stratum corneum · dermal–epidermal junction · dermal collagen · skin phototype · non-invasive diagnosis

Two-photon microscopy resolves living skin without stains or excision. Near-infrared femtosecond pulses excite fluorescence only where photon density is high enough for two photons to be absorbed at once, so signal arises from a sub-micron focal volume and optical sectioning is intrinsic rather than pinhole-derived.¹ In skin the same pulse train yields two complementary contrasts: two-photon excited fluorescence from keratin, NAD(P)H and FAD, melanin and elastin, and second harmonic generation from fibrillar collagen.^{2,3}

SYSTEM

All images were acquired with the SUPERVISION-780 In Vivo Two-Photon Microscopy Imaging System (SV780; TRANSVISTA, Nanjing, China).^{5,6} Excitation is by a 780 nm pulsed laser through a miniaturized handheld objective (NA 0.9, silicone oil or silicone gel immersion). The field of view is approximately $140 \times 140 \mu\text{m}$ and the axial scanning range exceeds $300 \mu\text{m}$, with a typical imaging depth of $120 \mu\text{m}$. Image stacks are acquired by stepwise axial scanning and collected at 512×512 pixels. Two-photon excited fluorescence (TPEF) and second harmonic generation (SHG) are detected simultaneously on two channels, TPEF at 420–580 nm and SHG at 375–405 nm.

Because the field is $140 \mu\text{m}$ across 512 pixels, in-plane sampling is $0.273 \mu\text{m}$ per pixel and the imaged area is $19,600 \mu\text{m}^2$. Every frame in this atlas carries a $20 \mu\text{m}$ scale bar drawn on that basis. A handheld geometry of this kind reaches histology-adjacent detail without a biopsy.¹⁰

WHAT EACH CHANNEL SHOWS

Channel A — fluorescence, 420–580 nm. Keratin dominates the stratum corneum; NAD(P)H and FAD in cytoplasm and mitochondria carry the granular and spinous layers; melanin is delivered to basal keratinocytes and carried outward with them as they differentiate, so it contributes to this channel throughout the epidermis and is why phototype is legible on two-photon at all.¹¹ In dermis the signal arises chiefly from elastin and, where present, elastotic material.^{3,4}

Channel B — harmonic, 375–405 nm. A coherent frequency-doubling process, not fluorescence, and in skin essentially specific to fibrillar collagen. It is absent from the epidermis, so the depth at which harmonic signal first appears marks the dermal–epidermal junction.^{2,7}

INDICES

DEJI is the junction surface area divided by its flat projection onto the $19,600 \mu\text{m}^2$ field, so a perfectly flat junction gives 1.000 and greater undulation gives larger values. **AF** and **SHG** are the projected areas of the segmented elastic and collagen fibre networks in the dermis, in μm^2 against the same field. Junction flattening is among the changes reported with intrinsic ageing on multiphoton imaging, alongside epidermal thinning and dermal elastosis.^{8,9}

Acquisition followed the instrument operation guide,⁵ at the level of detail needed to repeat the study.

SUBJECTS AND SITES

17 adult volunteers with clinically normal skin contributed the frames in this atlas, aged 23 to 68 years, Fitzpatrick types I to VI. Each was assigned a study number; age, sex and Fitzpatrick type were recorded at enrollment. Three sites were imaged in every subject, in the same order — left cheek, left forearm, back — spanning chronic sun exposure and habitual photoprotection within the same person. Subject, project and site were registered in the instrument software before imaging.

INSTRUMENT START-UP

The probe was inspected for a clean, undamaged window and intact contact between the silica gel and the window, and the four displacement-stage locking screws released with the supplied hex wrench. A session began only once every status indicator was lit.

COUPLING AND ZERO POINT

The probe was placed flat against the skin, avoiding hair follicles, visible vessels and lesional skin, and checked for light leakage. Under live imaging the displacement stage was driven with the long step (20 μm) until signal appeared, then the short step (1.00 μm) until the first sharp frame — the surface of the stratum corneum — was found. That frame was set as the zero point, so every depth in this atlas is referenced to the corneum surface rather than to the probe.

STACK ACQUISITION

Stacks were acquired in Z-stack mode from the zero point downward, both channels recorded simultaneously and excitation power ramped with depth.

Start position	0 μm	zero point, stratum corneum surface
Stop position	120 μm	guide limit is under 200 μm
Slice interval	2.00 μm	gives 61 optical sections per stack
Frames per slice	9	superimposed per layer
Frame size	512 × 512	140 × 140 μm, 0.273 μm/pixel
Initial power	400 mW	at the surface
Power step	20 mW/slice	ramp with depth
Maximum power	700 mW	ceiling for the ramp

FILES

Each stack is written as two multipage TIFFs, `ZStackFast_AF_Merge_date_time.tif` and `ZStackFast_SHG_Merge_date_time.tif`, retained unmodified as the archival record.

One slice is one optical section. Depth = (section – 1) × 2 μm, so section 1 is the surface and section 61 is ~120 μm. Depths carry a tilde because they are computed from section number, not measured: the 2 μm step, the operator-set zero point and compression under the probe each limit them to a few micrometres.

Layer assignment was made on morphology in the fluorescence channel. Thickness and the dermal indices were measured in the instrument's analysis module on the live stack.

LAYER ASSIGNMENT

Layers were assigned following published multiphoton criteria: keratin signal from anucleate squames in the stratum corneum; flattened polygonal cells with fluorescent cytoplasmic granules and dark nuclei in the granular layer; a honeycomb of polygonal keratinocytes with cytoplasmic NAD(P)H signal in the spinous layer, cell size falling with depth; basal keratinocyte rings around dermal papillae at the junction.⁴ The dermis is treated as a single compartment and is deliberately not subdivided into papillary and reticular.

THICKNESS MEASURES

Thickness was measured in the instrument's analysis module on the live stack, not derived from the sections reproduced here. Stratum corneum thickness was read manually at the corneum–granular transition. For the junction, the operator marked the first and last sections of the zone over which papillae and rete interdigitate; the depth of the first is the junction onset and the span between them is the junction zone. Both are reported in the appendix. A representative section chosen for a figure sits somewhere inside its layer rather than at the layer's boundary, so panel depths and these thicknesses are different quantities and should not be subtracted from one another.

DERMAL FIBRE AREAS

AF and SHG are the projected areas of the segmented elastic and collagen fibre networks returned by the analysis module over an operator-marked dermal window, in μm^2 against the 19,600 μm^2 field. The operator advances into the reticular dermis to the first frame in which fibre morphology is clearly resolved, marks it as the start frame, then advances a further five to ten frames to the end frame. These are areas, not intensities, and are not derived from the exported display frames.

COVERAGE OF THE MEASUREMENTS

Measurements were made on the live stack at acquisition; the image archive holds only sections later selected as representative. The two cover different acquisitions, which is why the counts behind the tables differ. Of 51 subject–site acquisitions, 45 contribute a corneum thickness to Table 4, 40 a junction measurement and 40 a complete dermal set to Table 5; one further acquisition was measured but is excluded throughout for marking sections beyond the stop position.

Where a measurement is absent it is because the stack would not support one: DEJI requires the junction contour traceable across the marked span, and the dermal indices require fibre networks the module can segment over twenty or more consecutive sections.

A section can be clear enough to reproduce while the stack around it is not clear enough to measure — several acquisitions here contribute an image but no measurement. Nothing was estimated, so the index values describe acquisitions good enough to measure rather than the series as a whole.

NOT MEASURED

Granular-layer cell density requires a separate marking step and was not recorded; it is omitted rather than approximated.

This atlas is a gallery, not a case series. Frames were chosen from the stacks on image quality and on how clearly each shows a named layer, then arranged so that one plate holds one layer and each plate runs from the youngest subject to the oldest.

SELECTION CRITERIA

Every acquisition was reviewed section by section. A section was eligible to be reproduced only if it satisfied all five criteria below. Criteria 1 and 2 are about the tissue; 3 to 5 are about the acquisition. A section failing any one of them was not used, however striking it appeared.

1	Unambiguous layer	The defining morphology of the named layer is present and cannot be confused with the layer above or below. The recognition criteria on each Figure A page are the ones applied.
2	Representative field	The morphology occupies the majority of the field rather than a corner. A section showing the layer only where a furrow happens to expose it was not used.
3	In focus, no motion	Cell borders or fibre outlines are resolved across the field. Sections with directional streaking from probe movement during the scan were rejected.
4	Even illumination	No probe-edge shadow, light leakage or gradient removing a usable portion of the field. Sections losing a third or more of the frame were rejected.
5	Traceable	The section number is recorded and the frame can be matched to its stack. A panel that cannot be traced to a section in the archive is not reproduced; Table 2 carries the section number for every panel printed.

HOW THE PANELS WERE THEN CHOSEN

Eligibility decides what may be shown, not what is shown. From the eligible sections, panels were selected to make three comparisons at each layer, and nothing else was reproduced.

The reference panel is the single clearest example of the layer in the whole series, chosen to be read alongside the recognition criteria.

The phototype ladder holds anatomic site constant and steps through Fitzpatrick type, so that the only variable between panels is pigment. Where no eligible section existed for a type at that site, the ladder stops rather than substituting a different site.

The site row is drawn entirely from one subject, so that between-person variation is removed from the comparison.

The age pair holds layer and site constant and contrasts the youngest and oldest eligible subjects available for that combination.

This atlas therefore shows the clearest available example of each thing it sets out to teach. It is a curated gallery, not a sample: the panels are not representative of the series, and the series is not representative of the population. Nothing in the figures is a prevalence or a group difference; the Limitations set out what follows from that.

Two pages per layer. The first teaches what the layer looks like and how to be sure you are at it; the second shows how that appearance changes with phototype, anatomic site and age.

PAGE A · RECOGNITION

One reference image, chosen as the clearest example of that layer in the series, beside the five features that identify it. The features are written as a decision sequence: they are the things to check, in order, before accepting that a section belongs to a given layer. Each page closes with the mistake most easily made at that depth.

PAGE B · VARIATION

Three strips, each isolating one variable. **Across phototype** holds anatomic site constant and steps through Fitzpatrick type, one subject per panel — it shows what pigment does to an image, not a difference between groups of people. **Across anatomic site** is drawn from a single subject, so the only difference between the three panels is where on the body they were taken. **Across age** holds both layer and site constant. The caption under each strip states what the strip is for and what can legitimately be concluded from it.

EVERY PANEL

Each panel is labelled with what it exemplifies — a phototype, a site, an age — and with the depth below the stratum corneum surface at which it was taken. Panels carry no study numbers: a reader should be comparing appearances, not subjects. Table 2 lists the site, demographic label and optical section behind every panel.

THE SCALE BAR

Every panel carries a white bar in its lower right corner labelled **20 µm**. The bar is not a border or a marker: its *length* equals twenty micrometres of tissue — 0.02 mm, roughly a quarter the width of a hair. It is printed because magnification means nothing once a picture is reproduced at an arbitrary size; the bar scales with the image, so the relationship holds. Every panel here is the same 140 × 140 µm field, so the bar is always one seventh of the panel width and is identical on every panel in the atlas. Use it to size what you are looking at: a spinous keratinocyte is about one bar across, its nucleus about half a bar, and a dermal papilla at the junction two to four bars wide.

DEPTH

Depth is the single most useful number on the page. The same layer sits at different depths in different people and at different sites, and reading the depth alongside the picture connects the two. All depths are $(\text{section} - 1) \times 2 \mu\text{m}$ below the stratum corneum surface, the zero point of every stack.

Every depth in this atlas is written with a tilde — ~24 µm, not 24 µm — and that is deliberate. Depth here is not measured; it is computed from the optical section number at the acquisition's slice interval. Three things blur it. The interval quantises depth to 2 µm steps, so no figure can be finer than that. The zero point is set by an operator judging the first sharp frame, which carries perhaps a section of uncertainty in either direction. And the probe compresses the skin it rests on, so the depth of a structure under the probe is not exactly its depth at rest. Treat every depth as accurate to a few micrometres, good for telling one layer from another and for saying which is deeper, and not for anything finer than that.

Every panel in this atlas comes from one of 17 volunteers with clinically normal skin, each imaged at the left cheek, left forearm and back. The table gives the composition of that series by age and phototype, which is what determines the comparisons the atlas can and cannot make.

AGE BAND, Y	FITZ I	FITZ I-II	FITZ II	FITZ III	FITZ IV	FITZ VI	ALL
23–29	2	•	1	3	4	1	11
30–39	•	•	•	•	1	•	1
40–49	•	•	•	•	•	•	•
50–59	•	•	1	•	•	•	1
60–68	1	1	1	1	•	•	4
All	3	1	3	4	5	1	17

Table 1. 12 of 17 subjects are female. Fitzpatrick V is not represented.

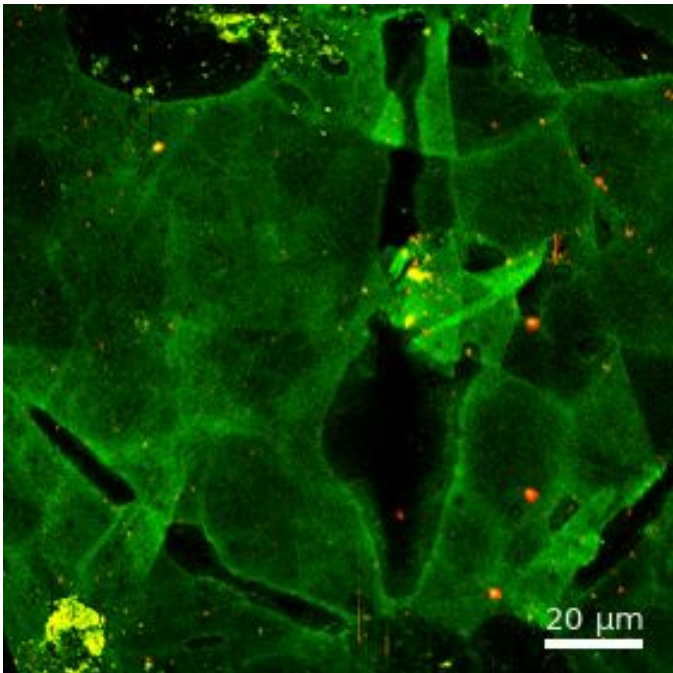
WHAT THIS COMPOSITION ALLOWS

Every phototype panel is one subject. A phototype strip shows one image per type at one anatomic site, so pigment rather than site is what differs down the row — but a strip of five panels is five people, not five groups. Read it as a demonstration of what pigment does to an image, never as a measured gradient or a comparison between phototype groups. Types I through IV are each represented by several subjects in the series, so a different panel could have been chosen; type VI is a single subject, type V is absent, and where a strip stops short it is because no eligible frame existed.

Site comparisons are made within one person. Every site row in this atlas shows cheek, forearm and back from the same subject at the same layer, which removes between-subject variation from the comparison entirely.

Age comparisons are illustrative only. 12 of 17 subjects are under 40 and the fifth decade is empty, so the age panels show what a difference looks like when it is present, not where in the lifespan it begins. Age and phototype are also not independent in this series: nearly every subject of type III or darker is under 40 and nearly every subject over 50 is type I or II. Where an age panel and a phototype panel appear to disagree, that confounding is the likeliest reason.

Stratum corneum



Reference Cheek · surface · Fitzpatrick III

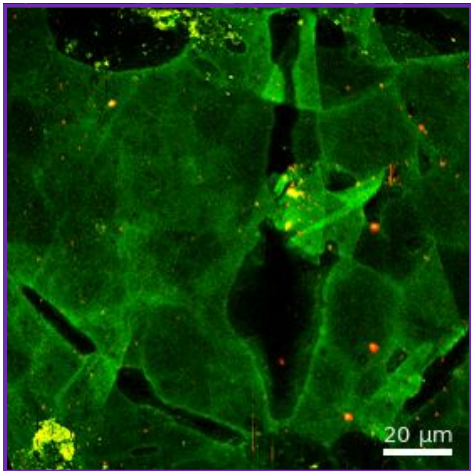
The reference appearance. Large anucleate corneocytes tessellate across the whole field, separated by the dark clefts of the skin furrows. Signal is uniform within each corneocyte and no nuclei are present.

WHAT IDENTIFIES THIS LAYER

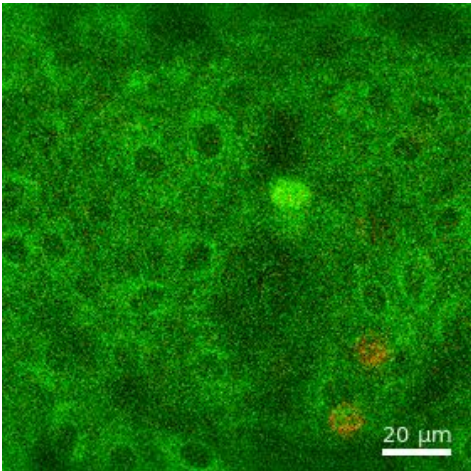
- 1 Anucleate corneocytes**
Broad polygonal corneocytes with no nucleus anywhere in the field. A visible nucleus means the section is already below the corneum.
- 2 Borders are furrows, not membranes**
The broad dark lines crossing the field are skin furrows, clefts in the surface rather than cell boundaries. Corneocyte outlines may also be visible; what distinguishes this layer is that no outline encloses a nucleus, and the corneocytes are larger and flatter than the cells of the granular mesh below.
- 3 Uniform keratin fluorescence**
Signal is flat and bright within each corneocyte, without the perinuclear granularity of the layer beneath.
- 4 Harmonic channel silent**
No collagen at this depth. Any appreciable harmonic signal means the section is at or below the junction, or an artifact is present.
- 5 Shallow**
Reached within the first few sections. If the corneum is not resolved by about 12 μm, suspect that the zero point was set below the surface.

COMMON PITFALL
Bright particulate material on the surface — sebum, residue from the coupling gel, exogenous particles — appears as punctate foci in both channels and should not be read as cellular structure.

WHERE IT SITS IN THE STACK the adjacent strata, superficial and deep



Stratum corneum surface

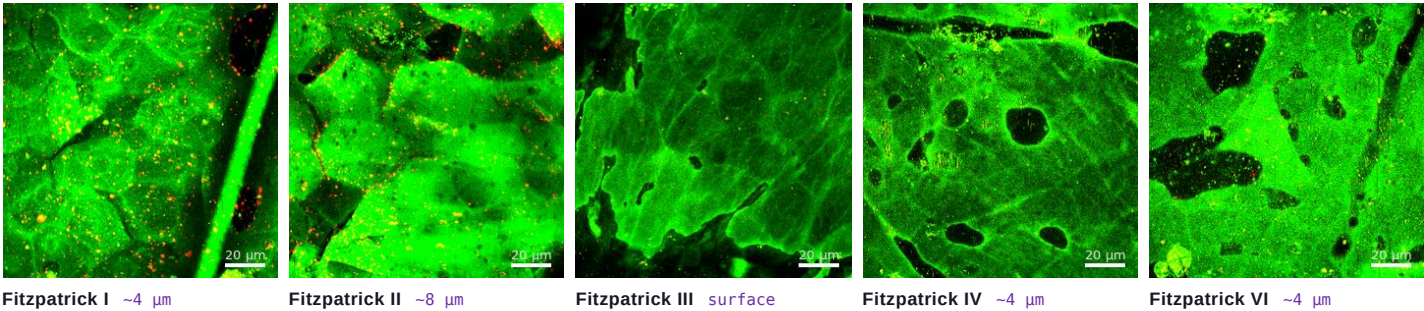


Stratum granulosum ~14 μm

Descending through the stack, each stratum gives way to the next over a few sections. Reading the strata immediately superficial and deep to a section is the quickest way to confirm which layer it belongs to.

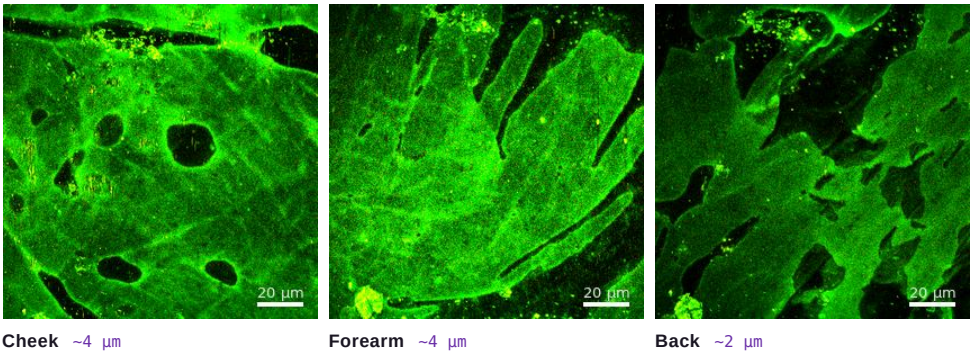
Stratum corneum

ACROSS PHOTOTYPE one anatomic site held constant



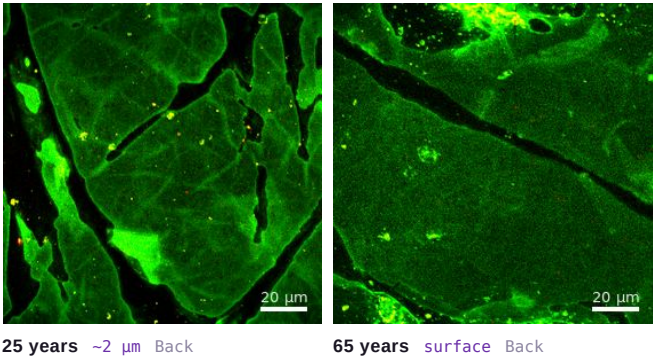
Cellular detail to compare: corneocyte outlines, furrow clefts, the absence of nuclei. All three are legible in every panel, and no cellular feature of this layer is lost as pigment increases. What does change is the texture within a corneocyte: the more pigmented panels carry fine punctate foci over the otherwise flat keratin signal. Melanosomes are carried upward from the basal layer as keratinocytes differentiate and reach the corneum itself in more pigmented skin, largely as isolated granules rather than the basal clusters of lighter skin,¹¹ and melanin is a strong two-photon fluorophore. Brightness cannot be compared between panels: display contrast is set per layer. Each panel is one subject.

ACROSS ANATOMIC SITE one subject, 24 years, Fitzpatrick IV



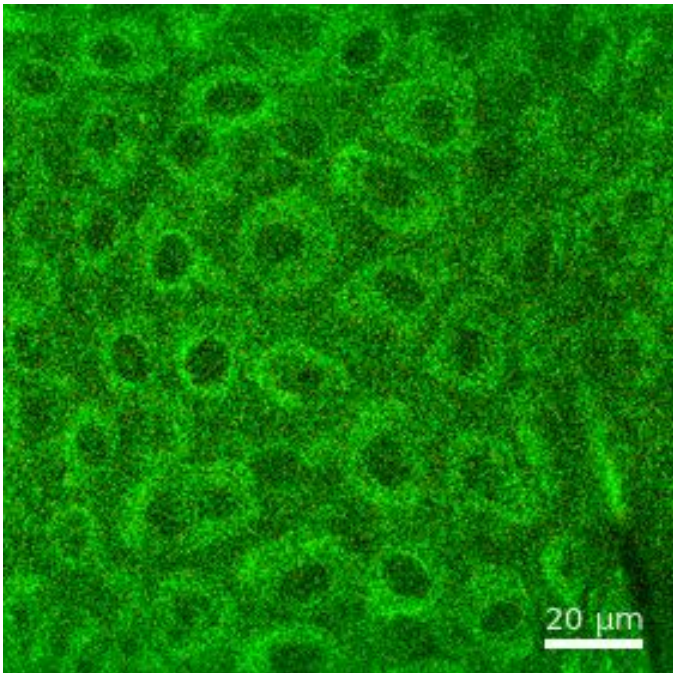
Furrow pattern and corneocyte size are the features to look at across these three panels. The cheek panel shows a finer furrow network and follicular ostia; the back panel the broadest corneocytes and coarsest furrows. All three are the same tissue at the same depth in one subject, so read them as three instances rather than as a site effect.

ACROSS AGE same layer, back



The architecture is the same in both panels: corneocytes broad and anucleate, furrows sharp. The furrows appear wider in the older panel. Corneocyte size and surface relief both change with age and neither can be separated from the other in a single section, so this pair shows the appearance and not its cause.

Stratum granulosum



Reference Cheek · ~14 μm · Fitzpatrick IV

The reference appearance. Large polygonal cells in an even mosaic, each with bright granular perinuclear cytoplasm surrounding a round, signal-poor nucleus.

WHAT IDENTIFIES THIS LAYER

- 1

First nucleated cells
The defining transition. Round dark nuclei appear against bright cytoplasm — the first depth at which discrete cells resolve.
- 2

Granular cytoplasm
Fluorescence is coarse and particulate around the nucleus, arising from NAD(P)H and FAD, with keratohyalin granules contributing to the granular texture. Flat, even cytoplasm suggests the corneum above.
- 3

Large, loosely packed cells
Granular cells are the largest nucleated keratinocytes in the stack, typically 25 to 35 μm. Cells that are smaller and tightly apposed are spinous.
- 4

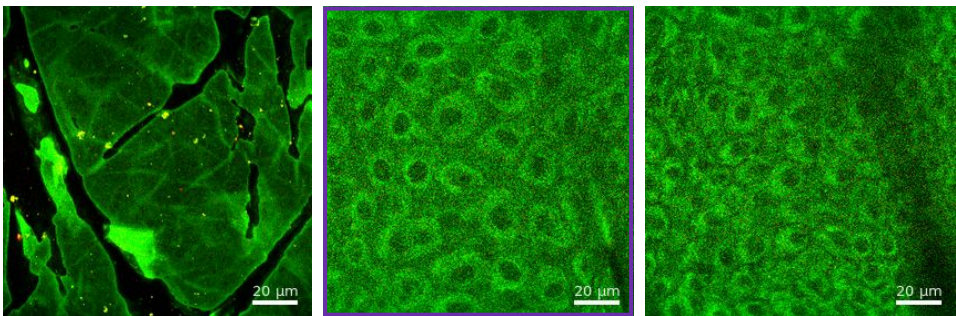
Abrupt transition
The change from corneum to granular layer happens over a few sections rather than gradually. A hybrid appearance usually means the section sits at the interface.
- 5

Harmonic still low
Collagen has not appeared. Harmonic occupancy is comparable to the corneum.

COMMON PITFALL

Skin furrows descend from the surface into this depth and cross the field as bright linear structures. They are surface relief carried down, not cell borders, and can obscure a third of the field.

WHERE IT SITS IN THE STACK the adjacent strata, superficial and deep

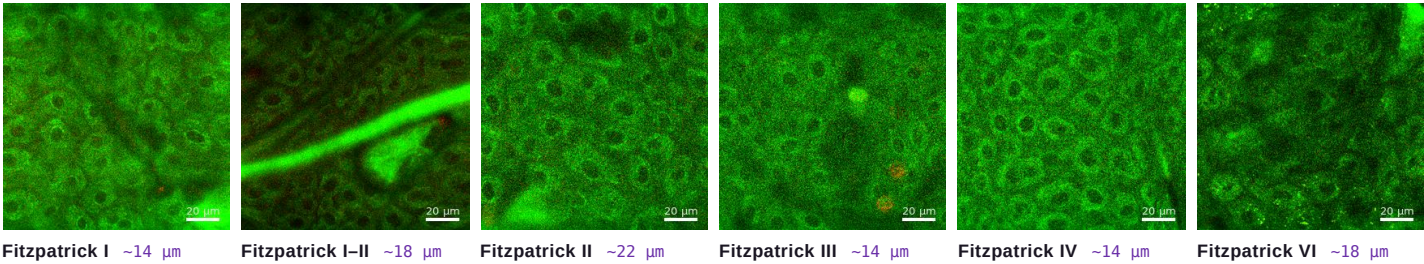


Stratum corneum ~2 μm Stratum granulosum ~14 μm Stratum spinosum ~30 μm

Descending through the stack, each stratum gives way to the next over a few sections. Reading the strata immediately superficial and deep to a section is the quickest way to confirm which layer it belongs to. The stratum corneum panel is from the same subject at the back.

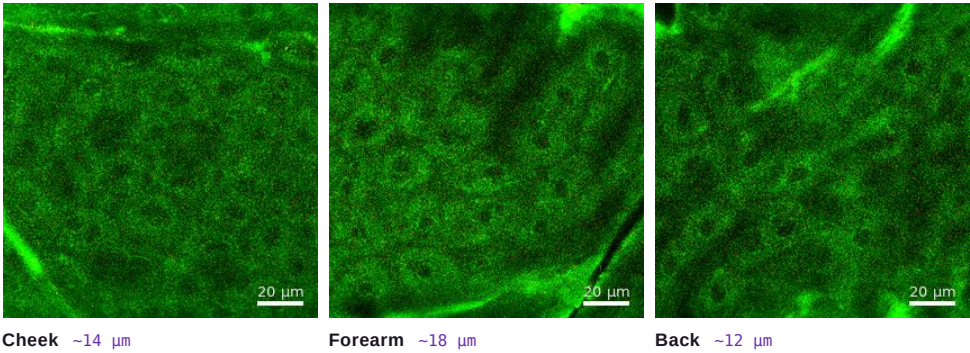
Stratum granulosum

ACROSS PHOTOTYPE one anatomic site held constant



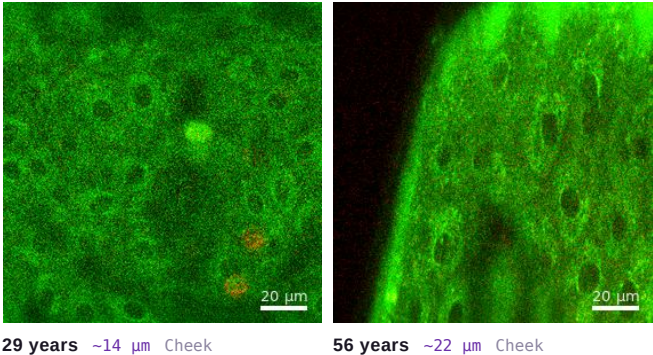
Cellular detail to compare: cell outlines, the dark round nucleus, the granular perinuclear cytoplasm. Every panel resolves all three. Cell size and packing look unchanged; what differs is the cytoplasmic texture, which carries additional bright punctate foci in the more pigmented panels and can make the perinuclear granularity harder to distinguish from pigment. Melanin is present through the whole epidermis rather than appearing at this depth, and it is a strong two-photon fluorophore, so it contributes here as it does above and below. Brightness cannot be read across these panels: display contrast is set per layer. Each phototype is one subject, type VI the only such subject in the series.

ACROSS ANATOMIC SITE one subject, 24 years, Fitzpatrick IV



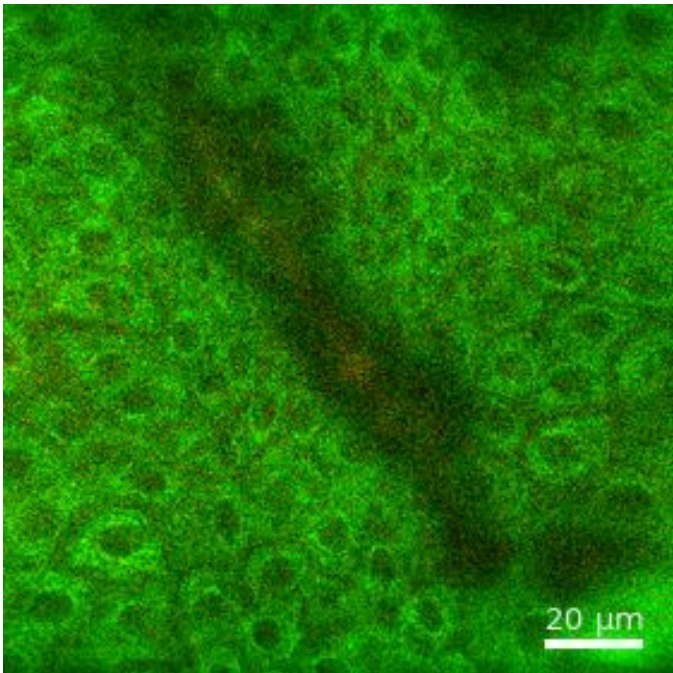
Cell size is the feature to look at across these three panels. Cell diameter was not measured in this series, so read the panels as three instances rather than as a site ordering; the epidermal thicknesses measured here run in the opposite direction, deepest at the cheek (Table 4).

ACROSS AGE same layer, cheek



Granular cells appear larger and more widely separated in the older panel. Keratinocyte and corneocyte size are reported to increase with age as turnover slows, but two panels cannot establish it and cell size was not measured here; the pair shows what the difference looks like when it is present.

Stratum spinosum



Reference Cheek · ~18 µm · Fitzpatrick I

The reference appearance. A regular honeycomb of polygonal keratinocytes, each with a bright cytoplasmic rim and a round nucleus of low signal intensity, filling the whole field.

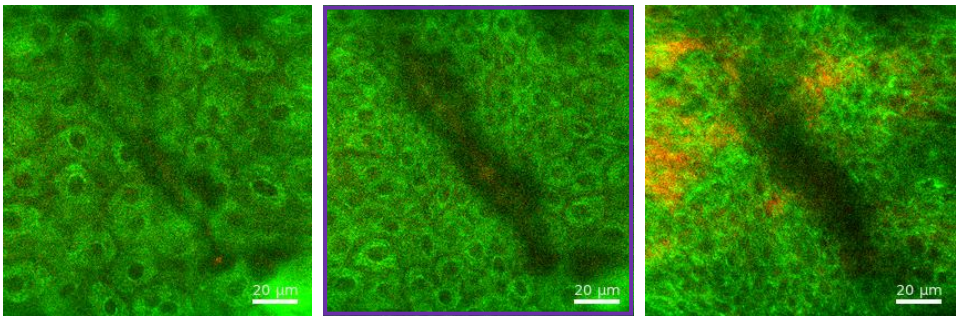
WHAT IDENTIFIES THIS LAYER

- 1 Honeycomb**
The single most reliable sign. Cells are polygonal, closely apposed, and tile the field with no gaps.
- 2 Bright rim, dark centre**
Cytoplasmic fluorescence forms a rim; the nucleus is signal-poor. The contrast between the two is what makes the honeycomb legible.
- 3 Smaller than granular**
Cell diameter is visibly less than in the layer above and continues to fall with depth through the layer.
- 4 No collagen yet**
Harmonic signal remains low. Its first appearance marks the junction below.
- 5 Even illumination expected**
A well-coupled spinous field is uniform edge to edge. Gradients usually indicate probe tilt rather than biology.

COMMON PITFALL

Graininess at this depth is common and is not the same as a poorly resolved honeycomb. Judge the cell pattern, not the noise: a grainy image in which every cell border can still be traced is usable.

WHERE IT SITS IN THE STACK the adjacent strata, superficial and deep

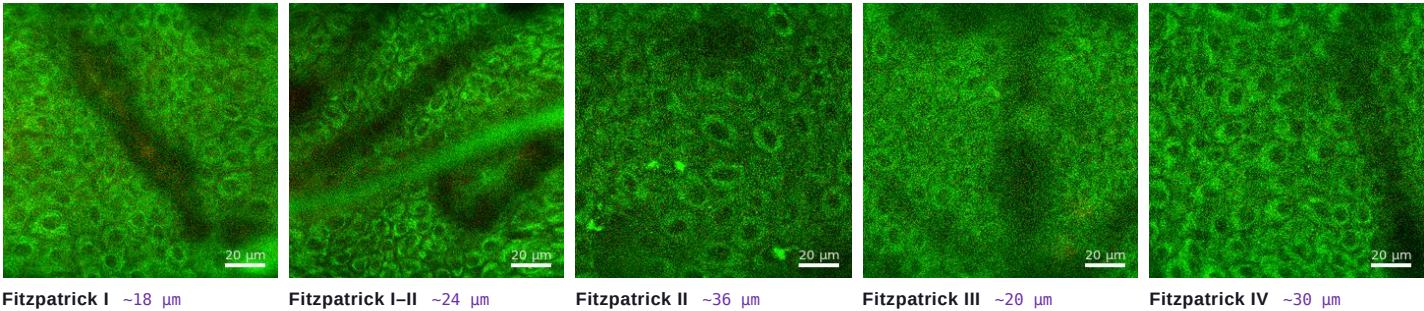


Stratum granulosum ~14 µm Stratum spinosum ~18 µm Junction ~28 µm

Descending through the stack, each stratum gives way to the next over a few sections. Reading the strata immediately superficial and deep to a section is the quickest way to confirm which layer it belongs to.

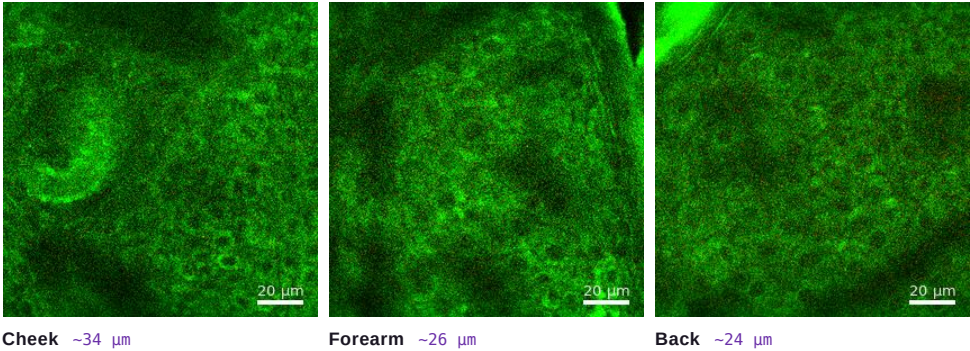
Stratum spinosum

ACROSS PHOTOTYPE one anatomic site held constant



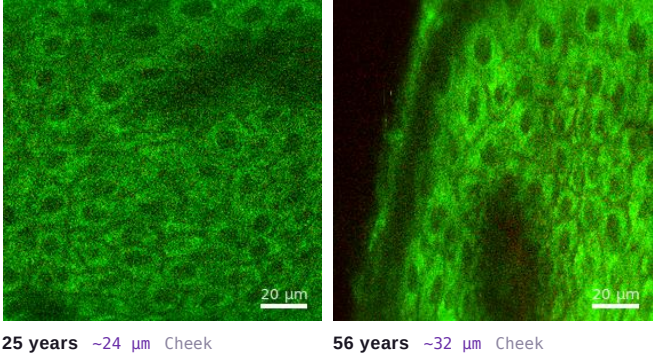
Cellular detail to compare: the honeycomb tiling, the bright cytoplasmic rim, the dark nucleus. The honeycomb is traceable in every panel. The rim is where the difference shows: in the more pigmented panels it carries a punctate quality from melanin within the keratinocytes, so the rim reads as granular rather than smooth, and the rim-to-nucleus contrast that makes the honeycomb legible is higher. Supranuclear melanin caps are described as a basal-layer feature, so what is seen at this depth is pigment carried upward with the differentiating cells rather than the cap itself. No type VI spinous frame reached publication standard in this series, so the ladder stops at type IV.

ACROSS ANATOMIC SITE one subject, 25 years, Fitzpatrick IV



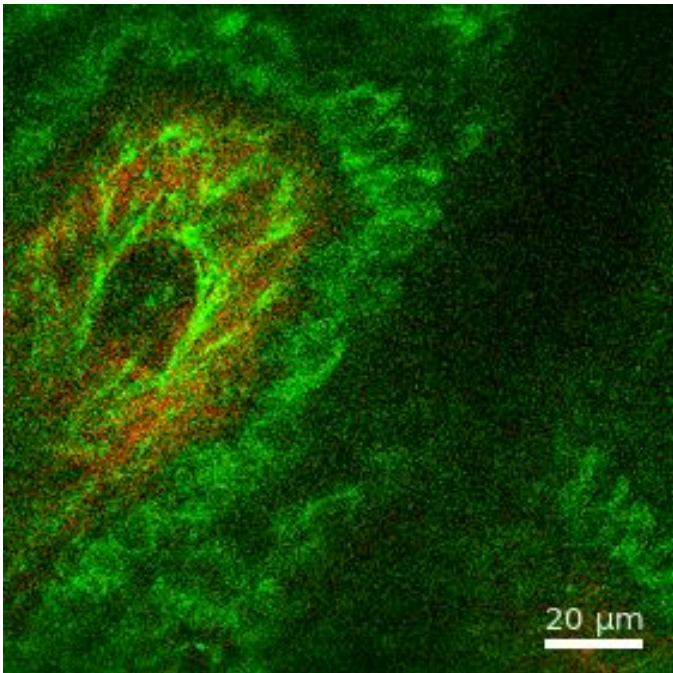
The honeycomb itself — rim bright, nucleus dark, cells tiling without gaps — is present in all three panels. Cell diameter appears to differ between them, but it was not measured in this series and these are three sections from one subject.

ACROSS AGE same layer, cheek



The honeycomb persists into the seventh decade. Cells are larger in the older subject and the pattern is slightly less regular, but the defining architecture does not change.

Dermal–epidermal junction



Reference Back · ~54 µm · Fitzpatrick III

The reference appearance. A dermal papilla sectioned near its apex: a ring of bright basal keratinocytes encircling a dark papillary centre, with harmonic collagen filling the core and a capillary loop running through it.

WHAT IDENTIFIES THIS LAYER

- Both channels carry structure**

Unique to this depth. Above it the harmonic channel is empty; below it the fluorescence channel loses cellular detail. Seeing cells and collagen in one field places the section at the junction.
- Basal rings**

Basal keratinocytes form bright closed rings around the papillae. A complete ring means the section is near the papilla apex; an open arc means it is off-centre.
- Dark papillary centres**

The papilla interior is signal-poor in the fluorescence channel and fills with harmonic signal instead.
- First collagen**

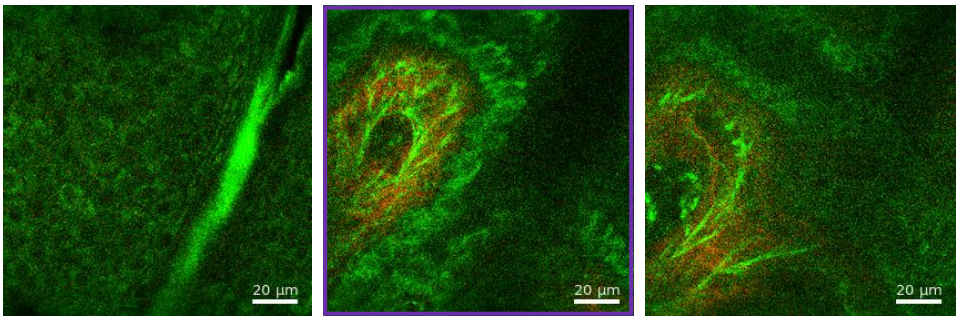
The depth at which harmonic signal first appears marks the base of the epidermis. Epidermal thickness in this atlas was measured from the operator-marked junction zone on the live stack, not from the section reproduced here.
- Capillary loops**

Signal-poor channels running through the papillary core, best seen when the section catches a papilla apex squarely.

COMMON PITFALL

A section a few microns too shallow shows basal rings with no harmonic signal and is a deep spinous frame, not a junction frame; a few microns too deep shows collagen with no rings and is papillary dermis. Both are common and neither supports a junction measurement.

WHERE IT SITS IN THE STACK the adjacent strata, superficial and deep

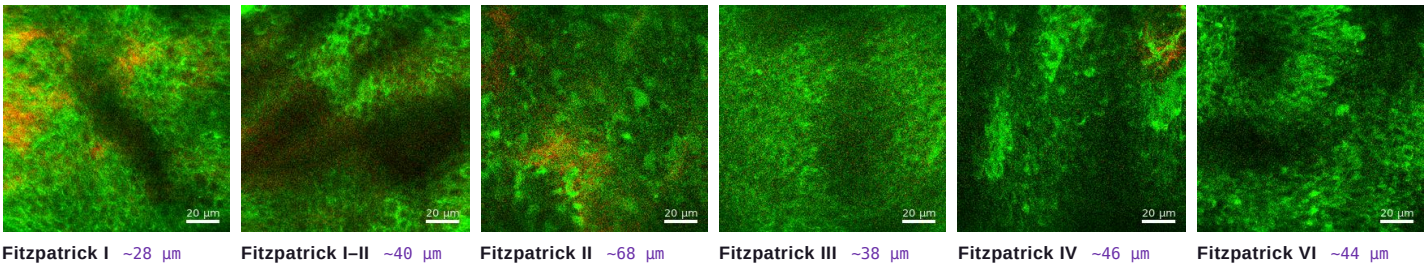


Stratum spinosum ~22 µm Junction ~54 µm Dermis ~82 µm

Descending through the stack, each stratum gives way to the next over a few sections. Reading the strata immediately superficial and deep to a section is the quickest way to confirm which layer it belongs to.

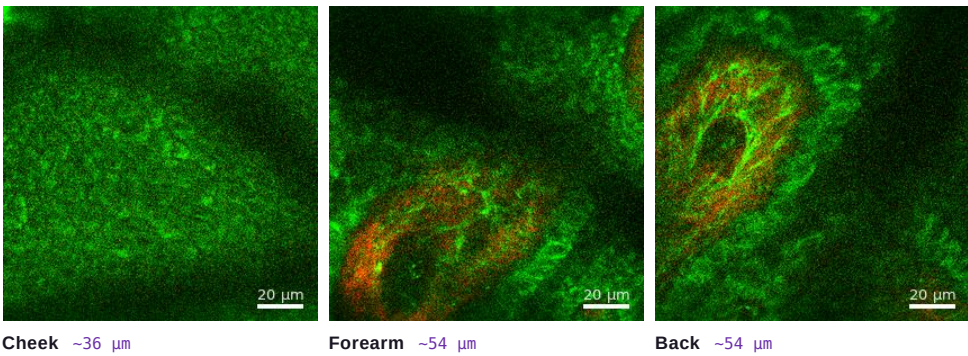
Dermal–epidermal junction

ACROSS PHOTOTYPE one anatomic site held constant



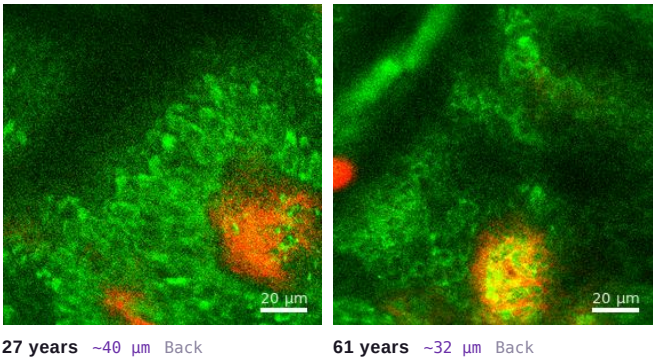
Cellular detail to compare: the ring of basal keratinocytes, individual cell outlines within that ring, and the dark papillary centre. This is the depth at which pigment changes cellular detail most. Melanosomes are delivered to basal keratinocytes and melanin is a strong two-photon fluorophore, so in the more pigmented panels the ring is the brightest structure in the field — bright enough that individual basal cell outlines within it become harder to separate, while the architecture around them, papillary spacing and ring diameter, looks unchanged. It is one subject per type, and type VI is a single subject, so read the sequence as a demonstration of the mechanism and not as a measured gradient.

ACROSS ANATOMIC SITE one subject, 24 years, Fitzpatrick III



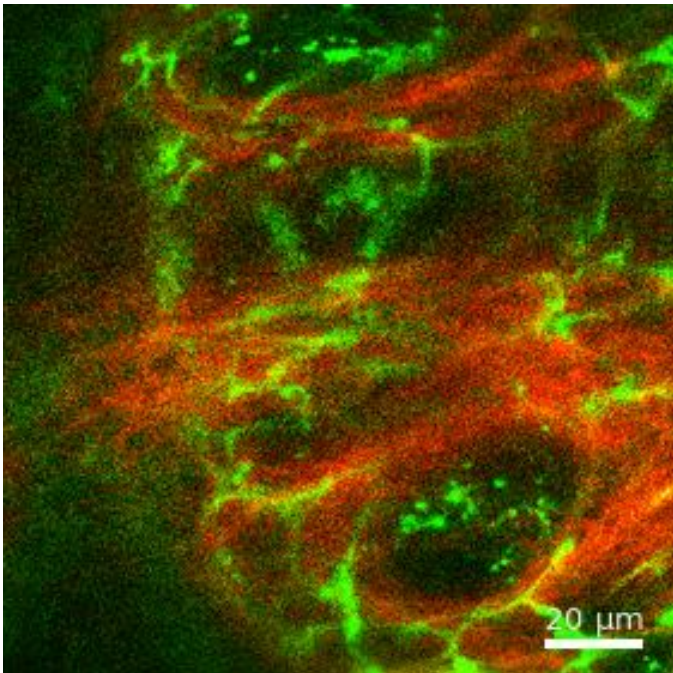
Papillary spacing and depth are what change across sites. The junction is reached at similar depth at all three sites in this subject; papillae are more closely spaced at the cheek.

ACROSS AGE same layer, back



Junction flattening is the feature to look for. The younger panel shows tightly interdigitating papillae with well-formed rings; the older panel a flatter contour with the rings more widely spaced. Flattening is among the changes reported with intrinsic ageing on multiphoton imaging,^{8,9} alongside epidermal thinning and dermal elastosis.

Dermis



Reference Forearm · ~62 µm · Fitzpatrick I

The reference appearance. Dense fibrillar collagen fills the harmonic channel in broad curving bundles, with bright elastic fibres running between and across them in the fluorescence channel.

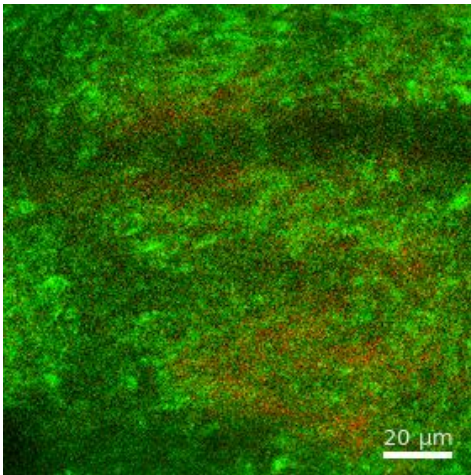
WHAT IDENTIFIES THIS LAYER

- 1 Harmonic dominates**
Collagen fills much of the field here, against effectively none above the junction. Segmented collagen area in this series ran from 2 to 21 per cent of the 19,600 µm² field, median 6 per cent (Table 5); what identifies the layer is that the harmonic channel carries continuous fibrillar structure at all. If it is sparse or empty, the section is still at or above the junction.
- 2 Two fibre populations**
Collagen in the harmonic channel, elastic fibres in the fluorescence channel. Their interweaving is the structure to read.
- 3 No cellular architecture**
Keratinocyte outlines are gone. Any honeycomb means the section is above the junction.
- 4 Irregular bundle orientation**
Normal dermal collagen runs in several directions at once. A single dominant orientation is a feature of scar, not of normal skin.
- 5 Adnexal structures**
Follicles and vessels appear as signal-poor channels with collagen curving around them, and are a useful landmark for depth.

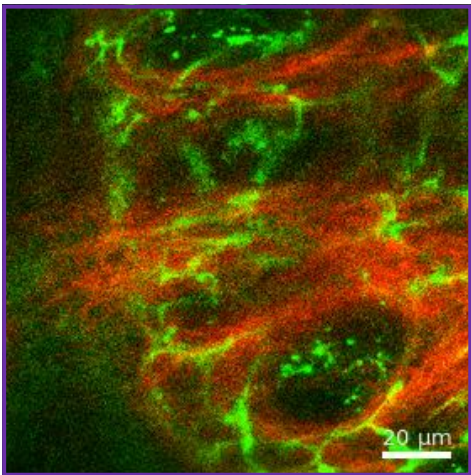
COMMON PITFALL

Because excitation power ramps with depth, deeper dermal frames are acquired at higher power and look brighter. Brightness here carries no biological meaning and must not be compared between panels.

WHERE IT SITS IN THE STACK the adjacent strata, superficial and deep



Junction ~32 µm

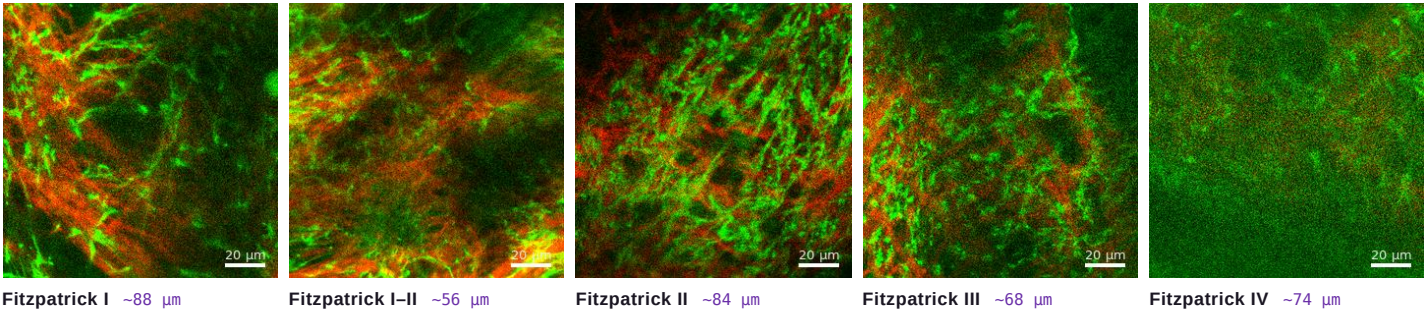


Dermis ~62 µm

Descending through the stack, each stratum gives way to the next over a few sections. Reading the strata immediately superficial and deep to a section is the quickest way to confirm which layer it belongs to.

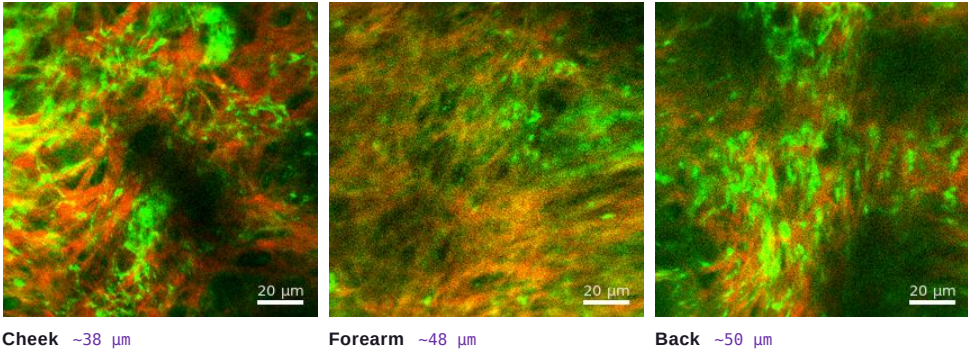
Dermis

ACROSS PHOTOTYPE one anatomic site held constant



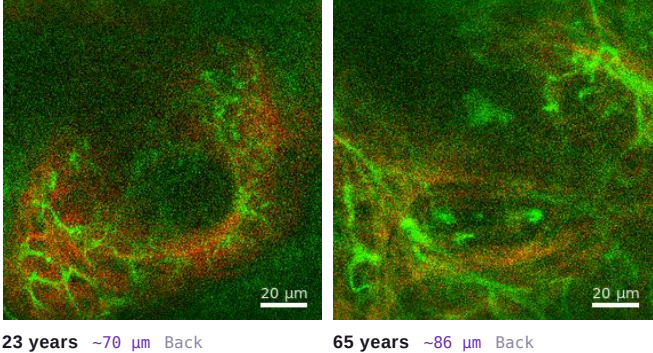
Detail to compare: collagen bundles in the harmonic channel, elastic fibres in the fluorescence channel. There are no cells at this depth; fibre detail is what changes. Second harmonic signal is generated at 390 nm, which melanin absorbs strongly, so collagen returning through a more pigmented epidermis is attenuated: the harmonic channel is weaker in the more pigmented panels despite comparable fluorescence, and individual bundles are harder to trace. This is an imaging effect, not a difference in the dermis, and worth knowing before any intensity-based measure is quoted in pigmented skin. No type VI dermal frame reached publication standard, so the ladder stops at type IV.

ACROSS ANATOMIC SITE one subject, 60 years, Fitzpatrick I



Fibre calibre and packing are the features to compare. The three panels come from one subject at the same depth band.

ACROSS AGE same layer, back



Both panels are back skin, the most photoprotected of the three sites. In the older panel the elastic fibres are thicker and brighter and run in coarser bundles, with patchier harmonic signal between them; the younger shows a finer, more evenly distributed elastic network on a more continuous collagen ground. Solar elastosis — broad amorphous fluorescent material displacing collagen outright — is a chronically exposed-site finding and is not what this pair shows.

Every panel reproduced in this atlas, with the site, demographic label and optical section behind it. Read down a figure to see which variable each strip holds constant. Depth is (section – 1) × 2 μm below the stratum corneum surface and is approximate throughout.

FIGURE	PANEL	SITE	AGE	SEX	FITZ	SECTION
STRATUM CORNEUM						
1A	Reference	Cheek	29	F	III	section 1 · surface
1B	Context · Stratum granulosum	Cheek	29	F	III	section 8 · ~14 μm
1B	Fitzpatrick I	Cheek	27	F	I	section 3 · ~4 μm
1B	Fitzpatrick II	Cheek	29	F	II	section 5 · ~8 μm
1B	Fitzpatrick III	Cheek	68	M	III	section 1 · surface
1B	Fitzpatrick IV	Cheek	24	F	IV	section 3 · ~4 μm
1B	Fitzpatrick VI	Cheek	24	F	VI	section 3 · ~4 μm
1B	Cheek	Cheek	24	F	IV	section 3 · ~4 μm
1B	Forearm	Forearm	24	F	IV	section 3 · ~4 μm
1B	Back	Back	24	F	IV	section 2 · ~2 μm
1B	25 years	Back	25	F	IV	section 2 · ~2 μm
1B	65 years	Back	65	M	II	section 1 · surface
STRATUM GRANULOSUM						
2A	Reference	Cheek	25	F	IV	section 8 · ~14 μm
2B	Context · Stratum corneum	Back	25	F	IV	section 2 · ~2 μm
2B	Context · Stratum spinosum	Cheek	25	F	IV	section 16 · ~30 μm
2B	Fitzpatrick I	Cheek	60	F	I	section 8 · ~14 μm
2B	Fitzpatrick I–II	Cheek	61	F	I–II	section 10 · ~18 μm
2B	Fitzpatrick II	Cheek	29	F	II	section 12 · ~22 μm
2B	Fitzpatrick III	Cheek	29	F	III	section 8 · ~14 μm
2B	Fitzpatrick IV	Cheek	25	F	IV	section 8 · ~14 μm
2B	Fitzpatrick VI	Cheek	24	F	VI	section 10 · ~18 μm
2B	Cheek	Cheek	24	F	IV	section 8 · ~14 μm
2B	Forearm	Forearm	24	F	IV	section 10 · ~18 μm
2B	Back	Back	24	F	IV	section 7 · ~12 μm
2B	29 years	Cheek	29	F	III	section 8 · ~14 μm
2B	56 years	Cheek	56	M	II	section 12 · ~22 μm
STRATUM SPINOSUM						
3A	Reference	Cheek	60	F	I	section 10 · ~18 μm
3B	Context · Stratum granulosum	Cheek	60	F	I	section 8 · ~14 μm
3B	Context · Junction	Cheek	60	F	I	section 15 · ~28 μm
3B	Fitzpatrick I	Cheek	60	F	I	section 10 · ~18 μm
3B	Fitzpatrick I–II	Cheek	61	F	I–II	section 13 · ~24 μm
3B	Fitzpatrick II	Cheek	29	F	II	section 19 · ~36 μm
3B	Fitzpatrick III	Cheek	29	F	III	section 11 · ~20 μm
3B	Fitzpatrick IV	Cheek	25	F	IV	section 16 · ~30 μm
3B	Cheek	Cheek	25	F	IV	section 18 · ~34 μm
3B	Forearm	Forearm	25	F	IV	section 14 · ~26 μm

FIGURE	PANEL	SITE	AGE	SEX	FITZ	SECTION
3B	Back	Back	25	F	IV	section 13 · ~24 μm
3B	25 years	Cheek	25	M	III	section 13 · ~24 μm
3B	56 years	Cheek	56	M	II	section 17 · ~32 μm
DERMAL–EPIDERMAL JUNCTION						
4A	Reference	Back	24	F	III	section 28 · ~54 μm
4B	Context · Stratum spinosum	Back	24	F	III	section 12 · ~22 μm
4B	Context · Dermis	Back	24	F	III	section 42 · ~82 μm
4B	Fitzpatrick I	Cheek	60	F	I	section 15 · ~28 μm
4B	Fitzpatrick I–II	Cheek	61	F	I–II	section 21 · ~40 μm
4B	Fitzpatrick II	Cheek	29	F	II	section 35 · ~68 μm
4B	Fitzpatrick III	Cheek	29	F	III	section 20 · ~38 μm
4B	Fitzpatrick IV	Cheek	27	M	IV	section 24 · ~46 μm
4B	Fitzpatrick VI	Cheek	24	F	VI	section 23 · ~44 μm
4B	Cheek	Cheek	24	F	III	section 19 · ~36 μm
4B	Forearm	Forearm	24	F	III	section 28 · ~54 μm
4B	Back	Back	24	F	III	section 28 · ~54 μm
4B	27 years	Back	27	F	I	section 21 · ~40 μm
4B	61 years	Back	61	F	I–II	section 17 · ~32 μm
DERMIS						
5A	Reference	Forearm	27	F	I	section 32 · ~62 μm
5B	Context · Junction	Forearm	27	F	I	section 17 · ~32 μm
5B	Fitzpatrick I	Cheek	27	F	I	section 45 · ~88 μm
5B	Fitzpatrick I–II	Cheek	61	F	I–II	section 29 · ~56 μm
5B	Fitzpatrick II	Cheek	29	F	II	section 43 · ~84 μm
5B	Fitzpatrick III	Cheek	29	F	III	section 35 · ~68 μm
5B	Fitzpatrick IV	Cheek	31	F	IV	section 38 · ~74 μm
5B	Cheek	Cheek	60	F	I	section 20 · ~38 μm
5B	Forearm	Forearm	60	F	I	section 25 · ~48 μm
5B	Back	Back	60	F	I	section 26 · ~50 μm
5B	23 years	Back	23	F	I	section 36 · ~70 μm
5B	65 years	Back	65	M	II	section 44 · ~86 μm

Table 2. 65 panels drawn from 17 of the 17 subjects in the series; no panel is reproduced twice. The plates and this table carry no study numbers. A separate frame index, held with the study record and not published, maps every panel to its subject and source file.

Two tables. The first gives the depth at which each layer was found across every section selected for the series; the second gives the layer thicknesses measured in the instrument’s analysis module. When a stack does not look the way a figure does, check the depth first. All values are approximate.

SITE	n	MEDIAN, μm	MIN, μm	MAX, μm	SD, μm
STRATUM CORNEUM					
Cheek	12	~4	0	12	3
Forearm	12	~3	0	6	2
Back	15	~2	0	6	2
All sites	39	~4	0	12	3
STRATUM GRANULOSUM					
Cheek	16	~17	12	46	8
Forearm	6	~18	14	20	2
Back	12	~16	12	18	2
All sites	34	~16	12	46	6
STRATUM SPINOSUM					
Cheek	13	~26	18	36	5
Forearm	4	~25	20	28	3
Back	9	~24	16	30	4
All sites	26	~24	16	36	5
DERMAL-EPIDERMAL JUNCTION					
Cheek	13	~44	26	76	14
Forearm	11	~42	26	54	9
Back	16	~44	28	60	10
All sites	40	~43	26	76	11
DERMIS					
Cheek	14	~85	38	110	17
Forearm	6	~76	48	86	13
Back	11	~70	50	90	13
All sites	31	~78	38	110	16

Table 3. Ranges overlap between adjacent layers: depth narrows the search but does not assign a layer — the recognition criteria on each Figure A page do that.

Layer thicknesses measured by the operator in the instrument’s analysis module, across every acquisition in the series rather than only the panels printed.

SITE	n	STRATUM CORNEUM, μm	RANGE	n	JUNCTION ONSET, μm	RANGE	JUNCTION ZONE, μm	RANGE
Cheek	15	~6	2–12	14	~27	16–40	~33	14–48
Forearm	14	~6	4–16	11	~24	16–36	~20	10–28
Back	16	~8	4–15	15	~22	14–34	~20	10–42
All sites	45	~6	2–16	40	~24	14–40	~22	10–48

Table 4. **Measurements, not panel depths.** These were made by the operator in the instrument’s analysis module across every acquisition in the series and are independent of the sections reproduced in the figures — which is why they do not match the depths printed on the panels. **Stratum corneum** is the measured thickness of the corneal layer. **Junction onset** is the depth of the first section of the operator-marked junction zone, and **junction zone** is the depth over which that zone extends, the interdigitation of papillae and rete. Corneum thickness was measured manually; three values are not multiples of 2 μm and were therefore read at a finer step than the section interval. One acquisition marking sections beyond the 120 μm stop position is excluded.

Stratum corneum is thinner at the cheek than at the back (median difference 2 μm, lower in 10 of 15 subjects, Wilcoxon $p = 0.007$) and than at the forearm ($p = 0.006$). Both are paired within subject, so between-person variation does not enter them.

The values these measurements took across this series of clinically normal skin, printed so a reader measuring their own acquisition has something to place it beside.

SITE	n	MEDIAN	MIN	MAX
DEJI — JUNCTION UNDULATION				
Cheek	14	1.031	1.012	1.035
Forearm	11	1.016	1.009	1.046
Back	15	1.016	1.010	1.052
All sites	40	1.020	1.009	1.052
AF — ELASTIC FIBRE AREA				
Cheek	14	2795	1348	4663
Forearm	11	1984	1665	3125
Back	15	2327	1503	3907
All sites	40	2420	1348	4663
SHG — COLLAGEN FIBRE AREA				
Cheek	14	1223	456	4146
Forearm	11	1044	541	3631
Back	15	1574	386	2312
All sites	40	1197	386	4146

Table 5. **DEJI** is the junction surface area over its flat projection; 1.000 is a flat junction and larger values more undulation. **AF** and **SHG** are the segmented elastic and collagen fibre areas in μm^2 against the 19,600 μm^2 field. All were measured by the operator in the analysis module on the live stack, over a marked junction zone and a marked dermal window; none can be derived from the exported display frames.

These are reference values, not norms, and the atlas makes no comparison between the rows. They rest on 40 of the 51 acquisitions in the series; the rest could not be measured, for the reasons set out in Methods. Three things move them independently of the tissue. They depend on the depth of the marked dermal window, which was not standardised here. They depend on how much pigment the returning signal passes through, since the harmonic channel is emitted at a wavelength melanin absorbs strongly — so a value from one phototype is not interchangeable with a value from another. And they depend on the segmentation the analysis module performs, which is specific to this instrument and software version. A reader should compare a measurement to these only when acquisition depth, phototype and software match.

A gallery teaches recognition. It does not establish how common an appearance is, or where in a range a change begins. These are the limits to hold while reading the figures.

THE PANELS ARE CHOSEN, NOT SAMPLED

Every panel is the clearest eligible section available for the comparison it illustrates — the right choice for teaching, the wrong one for inference. The atlas shows the legible end of what the instrument returns, not the average of it, so a trainee whose own stacks look less clean than these pages is not necessarily doing anything wrong. Nothing here is a prevalence, a mean, or a group difference.

The tables are selected too, by a different filter. A section can be clear enough to reproduce while the stack around it will not support a traced junction contour or a segmentable dermal window, so some acquisitions contribute an image and no measurement — more often where the image review also scored the acquisition poorly. The index values therefore describe acquisitions good enough to measure, not normal skin in general, and nothing was estimated to fill a gap.

THE POOL IS SMALL AND ITS AXES ARE CONFOUNDED

17 subjects contribute to five figures. Age and Fitzpatrick type are not independent here: nearly every subject of type III or darker is under 40, and nearly every subject over 50 is type I or II. The fifth decade is empty, sex is unbalanced, one subject represents type VI and none represents type V. The phototype strips should be read as *what pigment does to the image*, not as a comparison between groups of people; the age pairs likewise.

PHOTOTYPE DIFFERENCES ARE PARTLY OPTICAL

Melanin absorbs at the harmonic wavelength as well as fluorescing, so in more pigmented skin the collagen signal at depth falls while the fluorescence signal does not. Part of what the phototype strips show is melanin itself — supranuclear caps, brighter basal rings — and part is attenuation of the deeper channel by the pigment above it. The two cannot be separated from display frames.

SINGLE SECTIONS, NOT VOLUMES

Each panel is one optical section, not a projection through the layer, and the section shown is the clearest rather than the most typical.

DISPLAY PROCESSING

Brightness and contrast were adjusted for display, identically within each figure, using one window per layer — necessary because the acquisitions occupy only part of the available display range and the granular and spinous layers are intrinsically dim. Panel brightness carries no quantitative meaning and cannot be compared across figures. No frame was adjusted individually, cropped, resampled or retouched.

DEPTH RESTS ON THE ACQUISITION SETTING

Every depth printed is approximate and is written with a tilde for that reason: the 2 μm section step, the operator-set zero point and compression of the skin under the probe each move it by a few micrometres. 2 acquisitions carry section numbers beyond the stop position, probably from a different imaging mode, and are excluded from the tables and the figures. Archived frames carrying no section number appear nowhere here: an untraceable panel fails the fifth selection criterion.

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References 5 and 6 are manufacturer documents and are the only non-peer-reviewed sources cited. Reference 4 describes work using a device from the same manufacturer at the same excitation wavelength and is the source for the epidermal layer criteria in Methods.