InvVivo human skin imaging: Distinguishing cellular and fibrillar components with fluorescence lifetime microscopy

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TO THE EDITOR

Multiphoton microscopy has advanced clinical skin research by enabling in vivo 3-dimensional label-free imaging of human skin with submicron resolution and label-free molecular contrast. It is particularly effective for analyzing dermatological conditions (König, 2020; Lentsch et al, 2020; Shiu et al, 2024; Yew et al, 2014), including photoaging (Pena et al, 2022; Puschmann et al, 2012; Sugata et al, 2011), by assessing fibrillar structures such as collagen and elastin fibers through distinct spectral signals, specifically second harmonic generation for collagen and 2-photon excited fluorescence (TPEF) for elastin. However, evaluating cellular components in the dermis poses significant challenges, especially in photoaged or elastotic skin, where abnormal clumps and disorganized elastin structures can resemble cells. The weaker fluorescence signals from intrinsic fluorophores such as nicotinamide adenine dinucleotide phosphate and flavin adenine dinucleotide, combined with spectral overlap with elastin fluorescence, further complicate distinguishing these structures and limits specificity in identifying various cell populations. To overcome these challenges, we investigated in vivo fluorescence lifetime imaging microscopy (FLIM) to enhance molecular contrast. Although FLIM has been applied in other clinical skin-imaging contexts (Huck et al, 2016; Koehler et al, 2012; Seidenari et al, 2013), our study specifically focuses on its potential to distinguish cellular structures from fibrillar components. As a secondary finding, we investigated FLIM's capability to differentiate and quantify elastosis in human skin, proposing an index for this purpose as a rapid and accurate means of assessing photoaging, which warrants further exploration in future studies. By leveraging the fluorescent lifetimes of different molecular species, we aim to improve quantification of cell populations and extracellular matrix constituents, enhancing diagnostics and therapeutic strategies in dermatology.

To evaluate the effectiveness of clinical in vivo FLIM imaging for distinguishing cellular from fibrillar components in the human dermis, particularly against elastin fibers, we conducted imaging of 3 volunteers with varying degrees of elastosis. Images were acquired using a fast, large-area multiphoton exoscope, a multiphoton microscopy–based imaging platform with fluorescence-time resolved detection capabilities, developed in our laboratory (Supplementary Materials and Methods provides details). The images presented in Figure 1a were acquired from different participants or different locations within the same participant and were selected to represent scenarios exhibiting various distributions of abnormal elastin clumps that resemble cells. These images illustrate that distinguishing cellular structures from elastin clumps in the dermis is challenging or not possible when relying solely on intensity information (Figure 1a and Supplementary Figure S1a). This limitation highlights the need for enhanced imaging contrast and prompted our assessment of FLIM imaging to accurately identify and characterize dermal cells. In FLIM imaging, images are generated on the basis of fluorescence lifetime information, reflecting the time it takes for fluorescent molecules to decay. We used phasor analysis to assess fluorescence lifetime information (Supplementary Materials and Methods provides details), resulting in phasor plots (Figure 1b) that visually represent fluorescence decay at each pixel, along with phasor-mapped images (Figure 1c) that color code intensity images according to phase lifetime (angle) distribution. These phasor-mapped images revealed cellular-like structures exhibiting varying phase lifetimes, which served as the basis for distinguishing between cellular and elastin components through their distinct phase lifetime signatures. Cell-like structures exhibiting longer phase lifetimes were identified as elastin clumps, as demonstrated by their identical color coding to that of established elastin fibers in the phasor-mapped images (Figure 1c). Conversely, structures displaying shorter phase lifetimes were classified as cellular components. An expanded view of these structures and their corresponding phasor plot distribution is shown in Supplementary Figure S1.

The classification was further validated by segmenting cell-like structures (Figure 1d) and analyzing the phase lifetime values of all pixels in these regions of interest (Figure 1e). The analysis consistently revealed 2 distinct distributions: a shorter phase lifetime (modes in the interval [0.86, 1.07]) corresponding to cellular structures and a longer phase lifetime (modes in the interval [1.36, 1.59]) associated with elastin clumps. These distinct lifetime distributions enabled reliable discrimination between cellular and elastin components across all volunteers.

Identifying the cells also enabled us to characterize them using 3 analysis parameters relevant to skin imaging applications: phase lifetime, diameter,

Abbreviations: FLIM, fluorescence lifetime imaging microscopy; TPEF, 2-photon excited fluorescence

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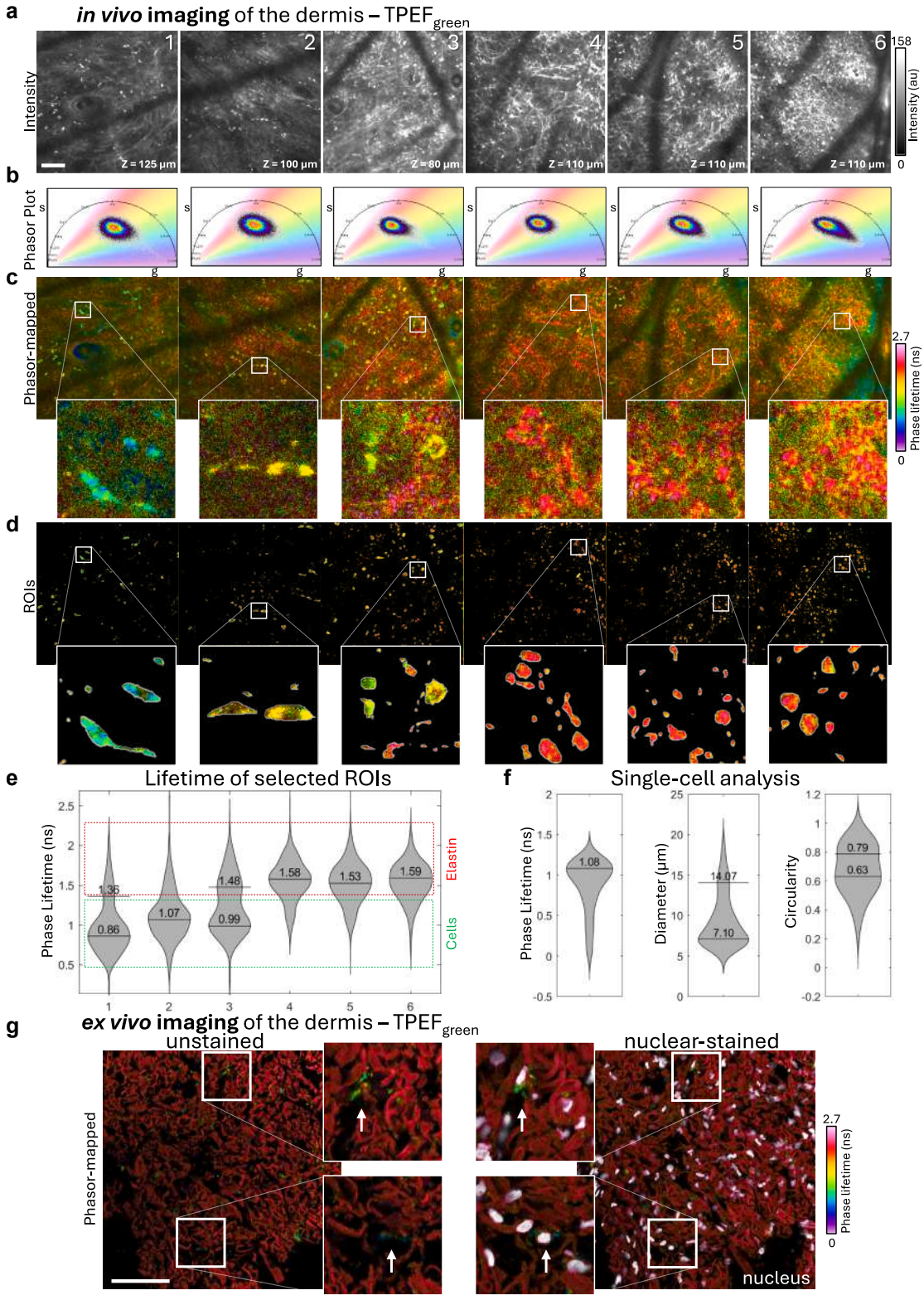

Figure 1. In vivo FLIM imaging distinguishes cellular and elastin components in human dermal skin. **(a)** In vivo intensity images ($TPEF_{green}$) from the forearm dermis of 3 volunteers (1−3) and from locations within the dermis of volunteer 1 (4−6). **(b)** Corresponding phasor plots for each image. **(c)** Corresponding color-coded phasor-mapped images. Insets highlight representative examples of cells and elastin clumps, with varying phase lifetimes, sizes, and shapes. **(d)** ROIs generated by segmenting cell-like structures. **(e)** Violin plots illustrate the phase lifetime distributions for each pixel in the ROIs, with horizontal bars indicating the mode(s) of each distribution. These distributions enable the discrimination between cellular and elastin components across all volunteers. **(f)** Single-cell analysis evaluating the diversity of dermal cell populations, with violin plots showing the distributions of phase lifetime (ns), diameter (µm), and circularity (1 = round shape). Horizontal bars indicate the mode(s) of each distribution. **(g)** Validation of FLIM signatures of cells and elastin fibers was performed by ex vivo imaging of fresh human dermis (discarded tissue from surgery). The tissue was imaged sequentially: first unstained, then after incubation with nuclear dye

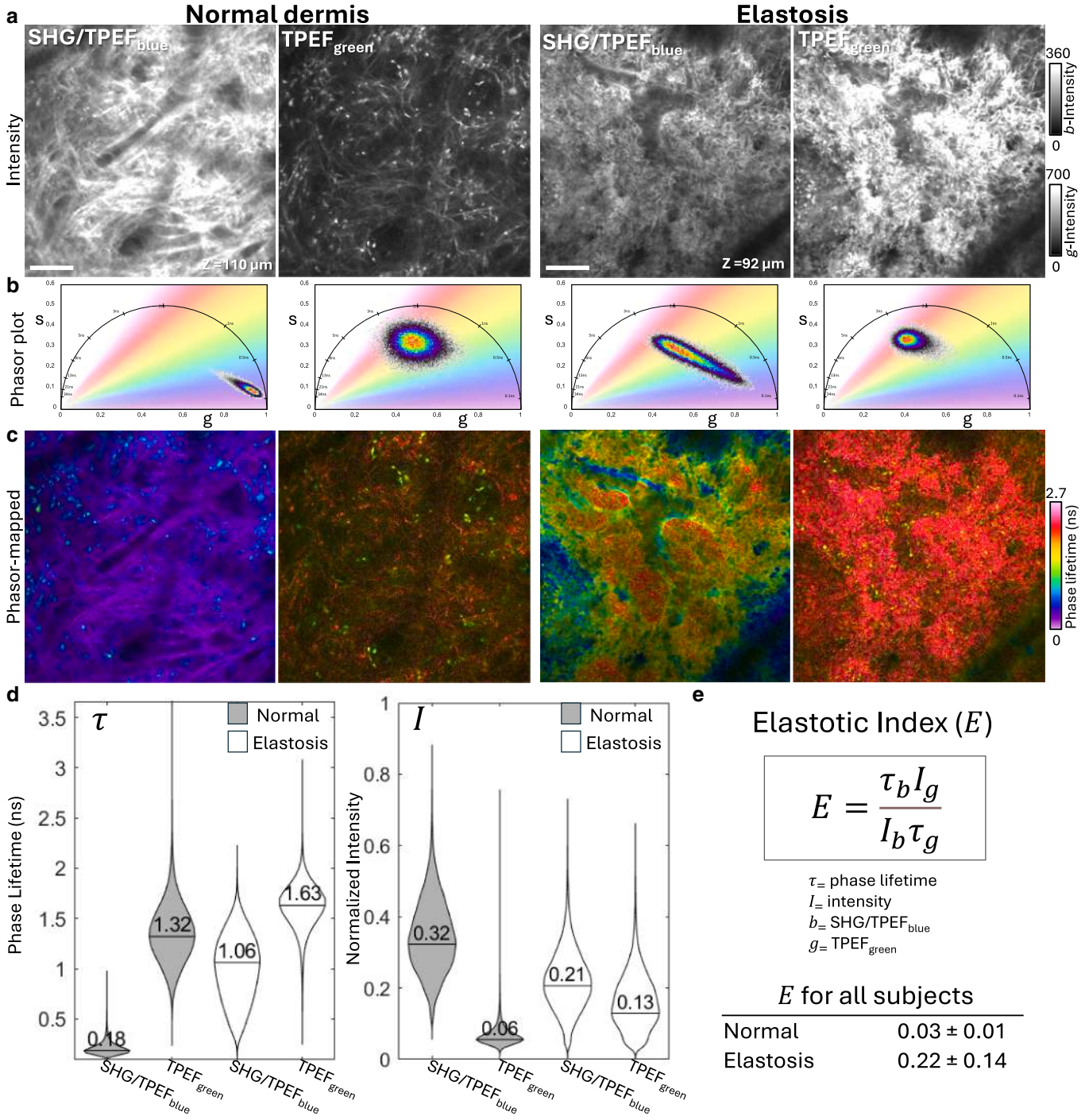

Figure 2. Quantification of elastosis using intensity and fluorescence lifetime information from elastin and collagen. **(a)** In vivo intensity images from the forearm dermis of 2 volunteers: 1 with normal dermis (aged 36 years) and 1 with elastotic dermis (aged 70 years), acquired on both detection channels— SHG/TPEF$_{blue}$ (*b*) and TPEF$_{green}$ (*g*). **(b)** Corresponding phasor plots for each image. **(c)** Corresponding color-coded phasor-mapped images. **(d)** Violin plots illustrate the phase lifetime and normalized intensity distributions for each pixel in the images from **c**, with horizontal bars indicating the mode(s) of each distribution. **(e)** Proposed elastotic index that uses the phase lifetime (τ) and intensity (*I*) data from both detection channels (*b* and *g*) discriminate between normal and elastotic dermis across all volunteers. A total of 6 participants were used to calculate elastotic index (*E*) mean values ± SD for normal (3) and elastotic skin (3). Bar = 100 µm. SHG, second harmonic generation; TPEF, 2-photon excited fluorescence.

and circularity. As an example, we used single-cell analysis to derive the values of these parameters for the segmented cells depicted in Figure 1. We found that most cells exhibited a phase lifetime mode value of approximately 1.1 ns, whereas size and shape showed broader distributions (Figure 1f).

(NucBlue) for cell identification. Representative color-coded phasor-mapped images of TPEF$_{green}$ are shown, demonstrating that cells have shorter lifetime than elastin. Bars = 100 µm. FLIM, fluorescence lifetime imaging microscopy; ROI, region of interest; TPEF, 2-photon excited fluorescence.

We confirmed that the shorter fluorescence lifetime structures are indeed cells, distinguishable from surrounding elastin and similar structures, through an ex vivo imaging experiment using fresh skin samples and subsequent nuclear staining (Figure 1g).

We further explored how FLIM can differentiate and quantify elastosis by in vivo imaging the dermal skin of 6 participants with varying degrees of photoaging. Figure 2a shows representative images comparing normal dermis with elastotic dermis in different individuals, captured using the fast, large-area multiphoton exoscope's detection channels: second harmonic generation/$TPEF_{blue}$ and $TPEF_{green}$. These images show higher intensity in the second harmonic generation/$TPEF_{blue}$ channel for normal dermis, whereas the $TPEF_{green}$ channel shows greater intensity for elastotic dermis, reflecting the abnormal accumulation of elastin at the expense of collagen breakdown in elastotic tissue. The color-coded phasor-mapped images (Figure 2c), generated from the corresponding phasor plots (Figure 2b), significantly enhance image contrast by highlighting changes related to the temporal decay of elastin and collagen fluorescence as well as alterations in the collagen second harmonic generation signal. We propose an index to quantify the presence of elastosis in the dermis that incorporates all information on intensity and temporal fluorescence decay from both detection channels (Figure 2e). Our results demonstrate that this index can reliably discriminate between normal and elastotic dermis across all volunteers (Figure 2d).

Our findings reveal, to our knowledge, a previously unreported capability of in vivo time-resolved 2-photon excited fluorescence imaging to differentiate between cellular and elastin components in human dermal skin. This capability is essential for the multiphoton microscopy imaging application in identifying, characterizing, and quantifying various dermal cell populations, thereby enhancing diagnostics and therapeutic strategies in dermatology. Cellular autofluorescence is not homogeneous across all cells; it varies depending on nicotinamide adenine dinucleotide phosphate and flavin adenine dinucleotide fluorescence, which are influenced by the cells' metabolic activity. Key parameters for characterizing these cells include fluorescence lifetime, which reflects different cell activity states, as well as size and shape, which are critical for distinguishing among various dermal and immune cell populations on the basis of morphological signatures. We also introduce a quantification index for evaluating elastosis and demonstrate its proof of concept. Although a previous study has suggested using fluorescence lifetime information for quantifying dermal elastosis (Koehler et al, 2012), our approach offers 2 potential advantages: (i) by integrating both intensity and temporal fluorescence decay data, it may improve robustness and sensitivity in detecting varying degrees of elastosis, and (ii) it may enable more efficiency for clinical imaging, featuring over a tenfold reduction in pixel dwell time and a larger scanning area, allowing better assessment of dermal morphological heterogeneities. Validation on a larger population is needed to confirm the extent of these benefits and the effectiveness of the index in clinical settings. In addition, it is important to note that fluorescence lifetimes can be influenced by microenvironmental factors such as cross-linking, hydration, viscosity, and pH as well as by various physiological and pathological conditions (eg, inflammation, fibrosis). Further studies evaluating these factors will be essential to fully understand the mechanistic basis of lifetime changes and to establish the technical limitations of this approach.

ETHICS STATEMENT

For in vivo imaging, all experiments were conducted with full written, informed consent of each participant under a protocol approved by the University of California, Irvine Institutional Review Board for clinical research in human subjects (HS# 2008-6307). For ex vivo imaging, we used fresh human skin tissue discarded from a surgery procedure (deidentified sample) under a protocol approved by the University of California, Irvine Institutional Biosafety Committee (UA-R277).

DATA AVAILABILITY STATEMENT

No large datasets were generated from these data. The datasets generated and/or analyzed in this study are available from the corresponding author upon request (mbalu@uci.edu).

KEYWORDS

Collagen; Dermal cells; Elastin fibers; Elastosis; FLIM

ORCID

Mihaela Balu: http://orcid.org/0000-0002-8018-5134

CONFLICT OF INTEREST

MB is a coauthor of a patent owned by the University of California, Irvine related to the development of clinical multiphoton microscopy technology. In addition, MB is a cofounder of Infraderm, LLC, a startup spin-off from University of California, Irvine focused on commercializing clinical multiphoton microscopy imaging platforms that may benefit from the use of advanced analysis tools. The Institutional Review Board and Conflict of Interest Office of University of California, Irvine have reviewed patent disclosures and found no concerns. The remaining authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: MB; Data Curation: BT, AV; Formal Analysis: BT; Funding Acquisition: MB, AKG; Investigation: BT, AFD, SR; Methodology: BT, SR, AV; Project Administration: MB; Resources: AFD, KMK, AKG, AD; Software: AV, PC, BT; Supervision: MB; Validation: BT; Visualization: BT, Writing — Original Draft Preparation: BT, MB; Writing — Review and Editing: BT, AV, SR, MF-H, AFD, AD, AKG, KMK, PC, MB

Belen Torrado[1], Alexander Vallmitjana[1], Suman Ranjit[1], Matthew Far-Hadian[1], Amanda F. Durkin[1], Alexander Dvornikov[1], Anand K. Ganesan[1,2], Kristen M. Kelly[1,2], Peter Chang[3,4] and Mihaela Balu[1,2,*]

[1]Beckman Laser Institute and Medical Clinic, University of California, Irvine, Irvine, California, USA; [2]Department of Dermatology, University of California, Irvine, Irvine, California, USA; [3]Department of Radiological Sciences, University of California, Irvine, Irvine, California, USA; and [4]Center for Artificial Intelligence in Diagnostic Medicine, University of California, Irvine, Irvine, California, USA

[*]Corresponding author e-mail: mbalu@uci.edu

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at 10.1016/j.jid.2025.10.612.

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SUPPLEMENTARY MATERIALS AND METHODS

Patient characteristics

A total of 9 volunteers (aged 31−69 years) were enrolled in this study. Imaging sites included the forearm and thigh.

Tissue staining

Nuclear staining was conducted using NucBlue reagent (Thermo Fisher Scientific). The tissue sample was incubated with a solution of 2 drops/ml of PBS 1X for 10 minutes at room temperature.

Clinical imaging device

Images were acquired using a fast, large-area multiphoton exoscope, a multiphoton microscopy−based imaging platform with fluorescence-time resolved detection capabilities, developed in our laboratory (Fast et al, 2020).

The specifications of this device have been previously described. Briefly, fast, large-area multiphoton exoscope uses excitation laser pulses at 785 nm, 80 MHz, 90 fs; 4 kHz resonant-galvo beam scanning module; custom-designed relay, beam-expander optics; a ×25 objective with numerical aperture = 1 and 60 mW laser power to image beneath the skin surface. The emission signal is captured by hybrid photodetectors on 2 channels: second harmonic generation/2-photon excited fluorescence$_{blue}$ (362−506 nm) and 2-photon excited fluorescence$_{green}$ (506−610 nm). Fast, large-area multiphoton exoscope incorporates fluorescence lifetime imaging capabilities using a 2.7G Hz digitizer to sample the analog signal from the photodetector, achieving a temporal resolution of 0.39 ns.

Image acquisition and analysis

Image acquisition is controlled by ScanImage software (MBF Bioscience, Ashburn, VA). Individual frames were captured at a resolution of 1200 × 1200 pixels, covering a skin area of 600 × 600 μm, at an effective rate of 4.5 s/frame for fluorescence lifetime imaging microscopy imaging.

Phasor analysis was performed using an open-source software platform, GSLab, recently developed by our laboratory (Vallmitjana et al, 2025). Coumarin 6 in ethanol was used for phasor plot calibration, which exhibits a monoexponential fluorescence decay time of 2.5 ns (Torrado et al, 2024).

Violin plots were constructed using kernel density estimation, with a threshold for mode(s) detection set at 10%. These plots were generated using custom scripts in MATLAB. Cell segmentation was performed using a semiautomated, custom-developed algorithm on the basis of U-Net, with a supervised postprocessing step. Single-cell analysis to extract morphological parameters was performed using custom scripts in MATLAB.

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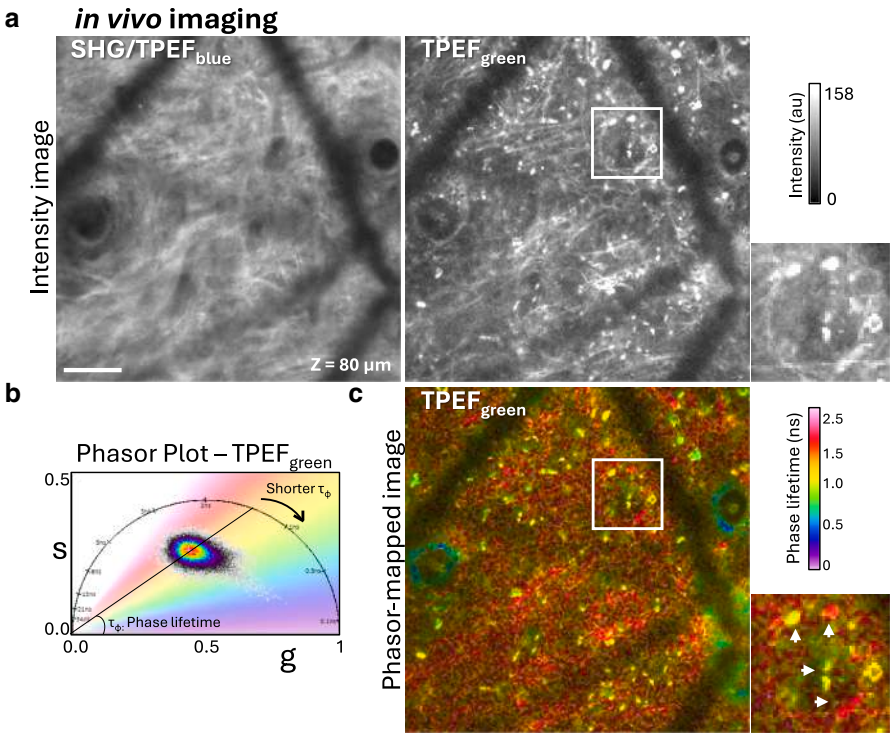

Supplementary Figure S1. FLIM-based differentiation of elastin clumps and cells in human dermal tissue. **(a)** Representative in vivo intensity images of human dermis acquired in SHG/$TPEF_{blue}$ and $TPEF_{green}$ channels. Inset highlights cell-like structures that are challenging to differentiate from other skin components solely on the basis of intensity information. **(b)** Corresponding phasor plot of the image captured on the $TPEF_{green}$ channel. **(c)** $TPEF_{green}$ intensity image color coded according to the phase lifetime distribution on the phasor plot. Inset highlights cell-like structures with heterogeneous fluorescence lifetimes. Bar = 100 µm. FLIM, fluorescence lifetime imaging microscopy; SHG, second harmonic generation; TPEF, 2-photon excited fluorescence.