Appendix S1.

MATERIALS AND METHODS

All human studies were approved by the authors' Institutional Review Board and followed the protocols of the Declaration of Helsinki. All subjects gave their written informed consent.

Sample preparation

Skin biopsies were taken from the volar forearm of 5 Caucasian males (median age 47 years; age range 40–57 years) with no history of skin disease themselves or among first-degree relatives. From each individual 2 skin samples were collected as shave biopsies. The skin samples were immediately put in 1-hexadecene (MERCK) to avoid dehydration during preparation for high-pressure freezing. Each sample was cut into pieces of 1×1 mm^2 with a razor-blade and placed in the cavity of a membrane carrier 1.5×0.1 nm^2 (Leica, Vienna, Austria) filled with 1-hexadecene. The skin samples were then immediately vitrified using a Leica EMPACT2 high-pressure freezer (Leica, Vienna, Austria). The samples were vitrified within 120 s after taking the shave biopsy.

CEMOVIS

For a general description of CEMOVIS see (21).

The vitrified pieces were removed from the membrane carriers and mounted in the cryo-chamber of a pre-cooled Ultracut S microtome (Leica, Vienna, Austria). The vitreous samples were trimmed using a diamond trimming knife (Diatome, Biel, Switzerland) and sectioned at –150°C with a nominal thickness of 30–50 nm using a 35° diamond knife (Diatome, Biel, Switzerland) with a clearance angle of 6°. The cutting speed was set to 0.2–1.0 mm/s. The sections were transferred to pre-cooled 1,000 mesh copper grids (Agar Scientific, Stansted, UK) using an eyelash glued on a wooden stick, and then pressed with a stamping tool. Grids with vitreous sections were placed in a 626 cryo-holder (GATAN, Pleasanton, CA, USA) at –180°C and inserted into a Philips CM200 FEG electron microscope (Philips, Eindhoven, The Netherlands). The accelerating voltage was set to 120kV. More than 1,000 images were recorded with a cooled slow scan $2,048 \times 2,048$ TVIPS TemCam-F224 HD CCD camera (pixel size 24 μm) at magnifications between 15,000 and 88,000, at a defocus between –0.5 and –3 μm, and with an electron dose between 3,000 and 13,000 e–/nm^2.

TOVIS

For a general description of TOVIS see (16).

The vitreous skin samples were sectioned at –150°C with a nominal thickness of 50 nm using a 35° diamond knife (Diatome, Biel, Switzerland) with a clearance angle of 6°. Sectioning was performed at a speed of 0.2–1.0 mm/s. The sections were transferred pre-cooled 600 mesh thin bar copper grids (Agar Scientific, Stansted, UK) and pressed with a stamping tool. Fiducial markers (PbS Core EviDots Espresso quantum dots (QDs), Evident Technologies, Troy, New York, USA) were deposited on the sections according to the method developed by (14). A facemask was used throughout the sectioning process and fiducial marker deposition procedure to minimize ice-crystal contamination on the section surfaces. Airflow, temperature (21°C) and humidity (<25%RH) were controlled at all times in the workroom.

The pre-cooled copper grids with vitreous sections were transferred to a pre-cooled 626 cryo-holder (GATAN, Pleasanton, CA, USA) at –180°C and inserted into a Philips CM200 FEG electron microscope (Philips, Eindhoven, The Netherlands), equipped with a cooled slow scan $2,048 \times 2,048$ TVIPS TemCam-F224 HD CCD camera (pixel size 24 μm) and software for automated data collection (TVIPS, Gauting, Germany). Tilt series ($n = 7$) from +60° to –60° with 1° increment (i.e. each tilt-series containing 120 images) were recorded at a defocus of –2 μm under low-dose conditions (in total approximately 4,000 e$^-$/nm^2 per 120 image tilt-series) at a magnification of 20,000 on an area of approximately $1,600 \times 1,700$ nm^2. Tomographic 3D-reconstruction by weighted back-projection, low-pass filtering to 15 Ångström and manual segmentation, were performed with the IMOD software package (28).

Freeze-substitution and embedding

For a general description of freeze-substitution of biological specimens see (33, 34).

High-pressure frozen skin samples were incubated in methanol containing 2% glutaraldehyde (GA) + 0.5% uranyl acetate (UAc) +1% OsO_4 at –95°C for 10 h, followed by –62°C for 8 h and –40°C for 4 h. After slowly heating the skin samples up to room temperature they were dehydrated in stepwise increased concentrations of acetone, embedded in LX-112 epon (Ladd Research Industrustries, Wiliston, Vermont, USA) and polymerized at 60°C.

FS-TEM serial tomography

Five sequential ultrathin serial sections (approximately 80 nm thick) were cut perpendicular to the skin surface from one and the same freeze-substituted skin sample. The sections (1 per grid) were collected on single-hole copper grids covered with Formvar (Agar Scientific, Stansted, UK). Sections were stained with 1% uranyl acetate and Sato lead stain (35). The grids were transferred to a Fishione 2,040 dual-axis tomography holder (Fishione Instruments Inc, Surrey, UK) and inserted into a Philips CM200 FEG electron microscope. Images were recorded with a pre-cooled slow scan $2,048 \times 2,048$ TVIPS TemCam-F224 HD CCD camera (pixel size 24 μm).

Five sequential dual axis tomograms, each composed of 240 images, were collected from +70° to –70° tilt with a magnification of 20,000 and a defocus of –0.5 μm, from an area of approximately $1,700 \times 1,600$ nm^2. Both single-tilts of the dual axis tomograms were combined and reconstructed in IMOD by weighted back-projection (36) into a single tomogram. Finally, the 5 sequential dual-tilt tomograms were aligned and then combined into a single serial-section tomogram.

The combined serial-section tomogram was then visualized by isosurface rendering, with the grey level set by using CEMOVIS post-tomography micrographs obtained at zero tilt angle from the same section area, as an internal reference. Membrane structures connected in 3D were segmented out using watershed segmentation (29). Isosurface rendering and segmentation were performed in USCF Chimera (30).

FS-FIB-SEM

For a general description of FIB-SEM see (17).

Using an ultramicrotome (Ultracut S microtome, Leica, Vienna, Austria) a small pyramid was prepared from each of 2 freeze-substituted skin samples. For each sample, a pyramid was cut from the skin sample block and mounted on a support stub of the FS-FIB-SEM, in a thick layer of conductive carbon, ensuring that the carbon made contact with the osmicated skin to improve conductivity. A 3-nm thick layer of platinum was deposited onto the sample with a sputter coater (HQ280; Cressington, Cressington Scientific Instruments Ltd, Watford, UK). The samples were transferred to a FEI Nova Nanolab600 Dualbeam (FEI, Eindhoven, The Netherlands). To protect the area

of interest from the ion beam and to prevent curtaining during cross-sectioning, a local 1-µm platinum layer was deposited on top of the sample. A U-shaped trench was milled at 30,000 eV accelerating voltage at an angle of 38° (17).

Subsequently the central part of the U-shaped trench was covered with a 750 nm-thick layer of platinum by ion beam-induced deposition. The sample was then rotated to 38° and a coarse surface was generated with a moderate current of 0.5–1 nA. To smooth the surface it was milled with a lower current of 50–300 pA. Slices were then milled in a sequential manner using an ion beam with a current of 0.3 nA and with a thickness of approximately 20 nm. The sample cross-sections obtained after each slice were then imaged in back-scatter electron mode using a 2,000 eV beam (0.21 nA) with a pixel-size of 3.57 nm. For each data-set, approximately 50 cross-sections were viewed with a dwell-time of 60 µs. In total, 3 FS-FIB-SEM data-sets were collected in 3 different areas from the 2 skin samples.

The stack of 50 2D images created with FS-FIB-SEM was combined to a single 3D volume using ImageJ (31) with the TomoJ plugin and manually aligned with IMOD (28). Lanczos re-sampling (32) was used to lower the resolution of the aligned image stack. The Lanczos kernel is a time-limited approximation to the sinc function. The sinc function is theoretically optimal to use for re-sampling of band-limited signals, but has infinite support, i.e. extends to infinity. In practice, Lanczos filtering can be seen as being more contrast preserving than Gaussian filtering, although some ringing artefacts can appear at strong edges in an image. Since our image data does not have discrete or very sharp edges at the original scale, Lanczos filtering is considered suitable. A re-sampled value is acquired by discrete convolution between the Lanczos kernel and the local image data around the position to be re-sampled. Re-sampling was performed with a down-sampling factor of 5.6.

The re-sampled volume was visualized using isosurface rendering. The settings for the grey level were determined by measuring an easily distinguishable feature, like a lamellar body, in the original 2D image. The same feature in the reconstructed 3D volume was made to fit the corresponding features in the original 2D images by setting correctly the grey-scale intensity value. All the connected features were segmented using watershed segmentation (see below). Both the isosurface rendering and segmentation were performed in UCSF Chimera (30).

Watershed segmentation

For a general description of watershed segmentation see (29).

Through computer-based image segmentation certain features can be extracted objectively from tomographic 3D-reconstructions and analysed separately.

We used the watershed algorithm integrated in Chimera (30) to segment out all the connected structures in the density maps of our FS-TEM serial tomography and FS-FIB-SEM data-sets. We first performed a Gaussian and a median filtering on the data-sets and determined the correct grey level by measuring several easy distinguishable features in the original 2D image. The same features in the 3D volume were made to fit the 2D measurements, by adjusting the grey-scale intensity value.

The watershed algorithm works as follows. If the considered voxel with a given density value is not adjacent to one or more voxels with the same or higher density value, it is assigned to a new region. When the considered voxel is adjacent to one or more voxels with the same or higher density value and the adjacent voxels are from 1 single region the considered voxel is assigned to that same region. If the considered voxel is adjacent to voxels from 2 or more regions, the regions are sorted by the number of adjacent voxels in each region in decreasing order; the considered voxel is then assigned to the highest region on the list. Thus the watershed algorithm traces by path growing all structures that are continuous with each other in 3D and assigns the same colour to them.