

Supplementary material: Investigating tattoo particles in the porcine model using X-ray phase-contrast tomography

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Micro-CT Data Processing

The acquired projection data sets at different modulator positions were processed using the modulation-tracking algorithm UMPA¹ in a runtime-optimized version². The algorithm tracks sub-pixel displacements (u_x, u_y) of the reference pattern on the detector face caused by sample-induced phase effects. To account for changes in the reference pattern over the course of the measurement, a dynamic reference correction was performed. This involved decomposing the full set of recorded reference images into eigenflats³ and finding the linear eigenflat combination that best matches a sample-free region in each projection^{4,5}. The sample-induced local pattern displacements compared to the dynamic reference image correspond to refraction angles (α_x, α_y) , and are converted to differential phase signals via

$$\left(\frac{\partial \phi}{\partial x}, \frac{\partial \phi}{\partial y} \right) = \frac{2\pi}{\lambda} (\alpha_x, \alpha_y) = (u_x, u_y) \frac{2\pi}{\lambda} \frac{p_{\text{eff}}}{z_{\text{eff}}}, \quad (1)$$

where (x, y) span a coordinate system on the detector face, $\left(\frac{\partial \phi}{\partial x}, \frac{\partial \phi}{\partial y} \right)$ are the x - and y -derivatives of the object's phase $\phi(x, y)$, λ is the X-ray wavelength, p_{eff} is the effective pixel size, and z_{eff} is the effective propagation distance between sample and detector¹. The phase (up to an additive constant C) is obtained through 2D Fourier integration^{6,7}

$$\phi(x, y) = \mathcal{F}^{-1} \left(\frac{\mathcal{F} \left(\frac{\partial \phi}{\partial x} + i \frac{\partial \phi}{\partial y} \right)}{ik_x - k_y} \right) + C, \quad (2)$$

where $\mathcal{F}$ and $\mathcal{F}^{-1}$ denote the Fourier and inverse Fourier transformations with respect to (x, y) . (k_x, k_y) are the corresponding Fourier-space coordinates. After setting $\phi = 0$ in sample-free regions, the projected electron density is recovered using

$$\rho_{e,\perp}(x,y) = \frac{-\phi(x,y)}{\lambda r_e}, \quad (3)$$

where r_e is the classical electron radius⁸. Finally, tomographic reconstruction of the projections yields the 3D electron density distribution using Xaid software (*Mitos GmbH*, Garching, Germany) with the Feldkamp-Davis-Kress (FDK) algorithm.

Human Skin Samples

The human biopsy with a blue ink, which also contains TiO_2 and CuPh, was investigated at the micro-CT endstation, see Fig. S1 (a). The sample is from a tattoo extending from the left thigh to the ankle joint, which showed an allergic reaction to the blue tattoo ink containing titanium dioxide (TiO_2) and CuPh. During the first weeks of the patient's second pregnancy, the most recently applied blue tattoos showed skin reactions with itchiness and plaques, while older sites tattooed with the same blue pigment reacted more weakly. The patient was diagnosed with contact dermatitis. A swelling of the skin can be observed more closely in Fig. S1 (b). While the epidermis has a slight swelling, the upper dermis has a more pronounced swelling, see white arrow. These swellings do not contain any tattoo particles themselves, yet are located directly above them. In Fig. S1 (c) blood vessels in the skin are demonstrated, further highlighting the capabilities of the system for the investigation of skin.

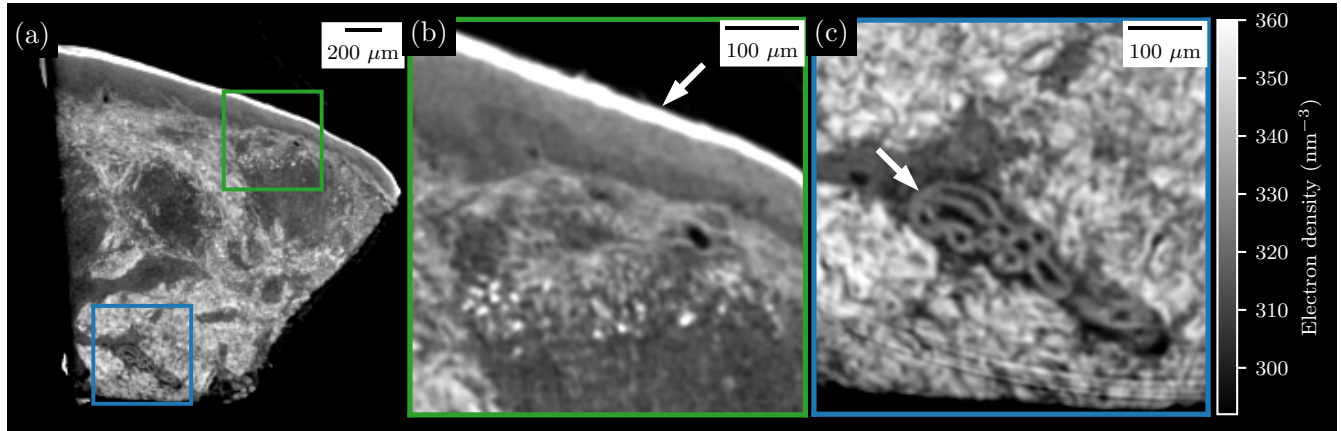


Figure S1. Images acquired using the micro-CT setup of a human skin biopsy with blue pigments (a). Zoomed-in regions are depicted in (b) and (c).

The human biopsy with an unknown organic red ink was investigated at the micro-CT endstation, see Fig. S2 (a). The tattoo was located on the right upper arm and contained an unknown organic red pigment. The patient exhibited two independent allergic reactions, a pseudolymphomatous and a granulomatous reaction, in 2013 and 2022. The biopsy was taken in 2023. The reaction, induced by the red pigment, was pronounced but differed markedly from that caused by the blue ink. The red pigment induced a strong immune reaction where immune cells gathered in lower regions of the dermis, see Fig. S2 (b, white arrows), and finally accumulated beneath the epidermis, Fig. S2 (c, black/yellow arrow). Furthermore, a strong swelling of the epidermal tissue is present.

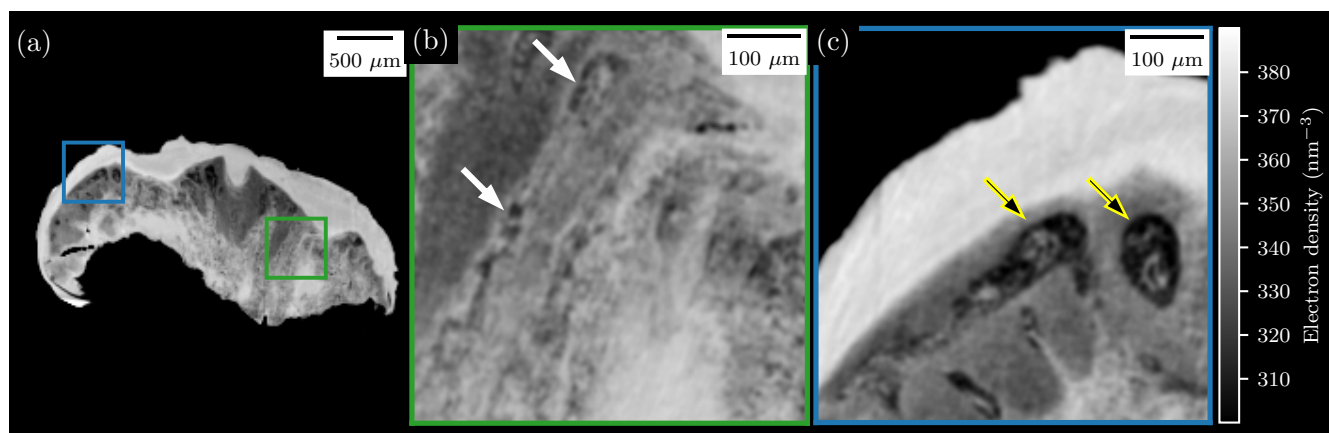


Figure S2. Images acquired using the micro-CT setup of a human skin biopsy with unknown organic red pigments (a). Zoomed-in regions are depicted in (b) and (c).

Pig skin renderings

Renderings were produced in Blender 5.2 (Blender Foundation, Amsterdam, the Netherlands) with the Cycles path-tracing engine on GPU (NVIDIA OptiX). Volumes were converted to OpenVDB grids and shaded with a Volume Scatter/Absorption material, mapping grey values to colour and opacity via a user-defined transfer function. Single images were rendered at 3840×2160 and animation frames at 1920×1080 , with Intel Open Image Denoise applied. Animations were rendered at 24 fps, frame-interpolated $5\times$ using RIFE (v4.26)⁹, and encoded at 60 fps.

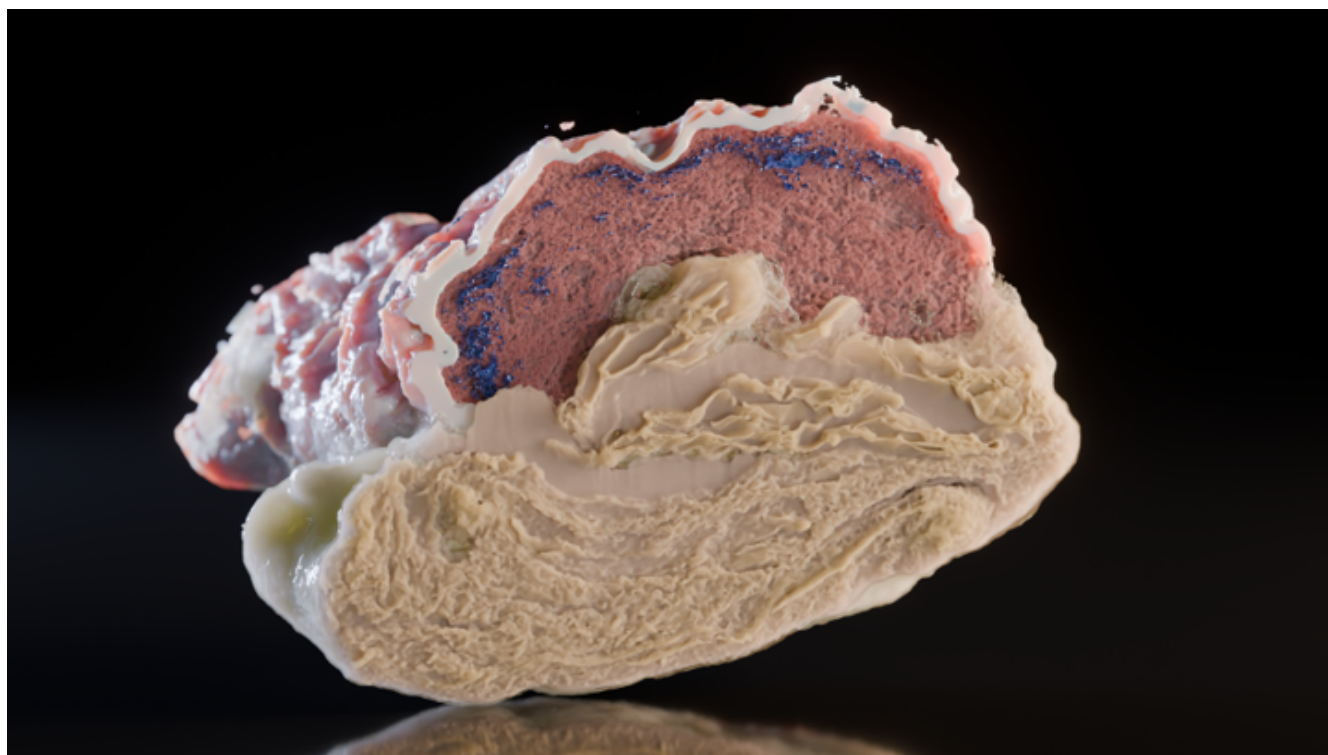


Figure S3. Rendered image of the tattooed pig skin at day 28. The tattoo particles are colored in blue.

In the rendered pig skin at day 28, the positioning of the tattoo particles is further highlighted. While no tattoo particles are visible in the epidermal layer, they agglomerate in the upper layer of the dermis.



Figure S4. Rendered image of the tattooed pig skin at at day 14. The tattoo particles are colored in blue.

The shedding of the tattoo particles becomes even more evident when the epidermis is rendered. Multiple tattoo particles are visible on the surface of the epidermis, where they will be subsequently shed.

Sample Preparation

micro- and nano-CT

The porcine skin samples were stored at -80°C and then fixed with 2.5 % glutaraldehyde. This fixation step preserves the tissue structure and prevents degradation during subsequent processing and imaging. The porcine biopsies were then dehydrated in a graded ethanol series, starting at 50 % and gradually increasing the concentration, to minimize tissue shrinkage and distortion. The samples were then placed in the clearing agent xylol for one hour. Next, the sample was embedded in paraffin at 62°C overnight, and the paraffin block was poured the following morning. After allowing it to harden for several hours on a cold plate, the sample was cut out of the paraffin block, and excess paraffin was removed. The paraffin enhances the contrast of the sample under X-rays, similar to a stain and provides the samples with structural support¹⁰. Sandpaper was used to smooth and round the surface, avoiding sharp cuts and edges to prevent artifacts.

The human biopsy with the allergic reaction to the red tattoo ink was embedded in paraffin at the hospital as part of clinical routine. The exact embedding protocol is not known, but it can be assumed to follow the same standard procedure as for the other biopsies.

TEM

Chemical fixation

The tissue samples were added to 1 ml of ice-cold fixative solution (25% glutaraldehyde [agar scientific] in $1\times$ PBS [Carl Roth]). The samples were incubated in a 2 ml centrifuge tube at 4°C to 6°C overnight.

Dehydration and Epon embedding

The fixated tissue samples were carefully removed from the centrifuge tube. The samples were dehydrated using ethanol in several concentrations (30 – 90, 96, 100% (v/v), each concentration for 1 h incubation time, Carl Roth). Finally, the tissue samples were transferred into 100% (v/v) absolute ethanol (Carl Roth), where they resided until Epon embedding, which was conducted in an embedding machine (Lynx II). All vials were filled with 20 ml of chemicals and reagents containing propylene oxide and Epon, or a mixture of both. The Epon contained the following chemicals and reagents, respectively: agar 100 resin (24 g, Agar 100 resin Kit, Agar Scientific), [3-(2-dodecenyl)succinic anhydride] (9 g, DDSA, Agar 100 resin

Kit, Agar Scientific), (Methyl-5-norbornene)-2,3-dicarboxylic anhydride (15 g, MNA, Agar 100 resin Kit, Agar Scientific) and bethyldimethylamine (1.4 g, BDMA, Agar 100 resin Kit, Agar Scientific). The Epon was freshly prepared and stirred on a magnetic stirring plate for 20 min before use. The dehydrated tissue samples were transferred into 20 ml propylene oxide where they incubated for 2×4 h. Then the Epon was introduced into the sample by increasing the Epon content stepwise using the following ratios of propylene oxide and Epon (v/v): 2:1, 1:1 and 1/2, respectively. Finally, the tissue samples were transferred into pure Epon (incubation for a minimum of 4 h) where they resided until further use. All vials were filled with 20 ml of chemicals and reagents.

Serial sectioning

The resin block was trimmed (Leica EM Trim, Leica), then serial sections of 70 nm thickness (diamond knife, ultra 35°, 2.00 mm DiATOME) were made using an ultramicrotome (Leica Ultracut UC T, Leica). Two to four consecutive sections were placed on a formvar-coated grid (Cu, 200 mesh, Plano GmbH). Several grids were prepared per sample and allowed to air-dry on a petri dish lined with filter paper.

Post staining

The post-staining was mainly conducted according to the protocol as outlined in Köhrer et al.¹¹. Throughout all the following steps, the grid floated on the surface of the drops during incubation, with the sections facing downward. Briefly, a parafilm was prepared with all reagents, whereby the size depended on the number of grids to be stained. As per grid, one row of reagents was used (one drop was ca. 25 μ L). First, the grid was placed for 1 min onto the methanol (50% v/v, Morphisto) drop, then transferred to uranyl acetate (2% (v/v) aqueous uranyl acetate (Electron Microscopy Sciences) in 70% (v/v) methanol (Morphisto) and incubated for 5 min. The parafilm was covered with a lid. The grid was rinsed in all 4 methanol (50% v/v (Morphisto) drops, then placed in the first water drop. The grid was incubated for 1 min with the lid on, then the process was repeated twice more before moving to lead citrate (Science Services) staining. Here, the lead citrate drop is placed next to a sodium hydroxide pellet (Carl Roth) to avoid oxidation of the lead(II). The grid was incubated for 3 min in lead(II) citrate (Science Services) before being washed four times with aqua bidest. Each washing step lasted 1 min. The grids were allowed to air-dry on a petri dish lined with filter paper. The individual grids were then subjected to TEM imaging.

Animal Procedures

This section is taken directly from Cambiasol *et al.*¹² as the samples stem from this work.

Preoperatively each animal received an intramuscular injection of 0.5 mg/kg Midazolam (Midazolam Accord, Accord Healthcare Limited, Devon, UK) 10 mg/kg Ketamin (Ketazol, Graeb, Bern care Limited, Devon, UK) 2 mg/kg Azaperon (Stresnil, Elanco GmbH, Bad Homburg vor der Höhe, Germany) and 0.2 mg/kg Butorphanol (Butomidor, Richter Pharma AG, Vienna, Austria). General anesthesia was subsequently performed with a standard tracheal intubation and 2 mg/kg/h to 5 mg/kg/h Propofol, 20 μ g/kg/h Fentanyl and 1 % to 2 % Sevofluran.

The euthanasia was performed during general anesthesia through intravenous Pentobarbital-Natrium injection (Euthasol, Virbac, Carros, France) on the last day of the study course.

After shaving, each animal was tattooed by a professional tattoo artist using a Cheyenne HAWK Pen (Cheyenne, Berlin, Germany), following standard hygiene procedures. Intenze Cleanze concentrate (Intenze Ink., Rochelle Park, New Jersey, USA) was used to cleanse the skin before skin disinfection and prior to tattooing. Bevor, during and after tattooing all areas were moistened with Purple Glide (Inkeeze, Newbury Park, California, USA).

Each pig was tattooed on the inner thigh of the anterior and posterior legs bilaterally. Four approximately 4×4 cm areas were tattooed. In addition, one control area of 4×4 cm of each posterior thigh was marked.

After tattooing, each leg was dressed with Mepilex® Safetac (Mölnlycke Health Care, Göteborg, Sweden) and Opsite® (Smith & Nephew, Watford, United Kingdom). All products used in the tattooing process underwent composition analysis to determine eventual cross-contamination. Pain management with Fentanyl patches of 25 μ g/h to 50 μ g/h (depending on individual needs) were administered.

After intravenous heparinization (200 IU pro kg) and euthanasia, the animals underwent a midline laparotomy. After identifying the aorta, the artery was prepared for cannulation direct under the arch. Once ligated, the perfusion catheter was inserted and the proximal aorta was cross-clamped and gravity perfusion with approximately 4 °C NaCl 0.9 % was performed. The homogenization of each organ was performed chopping with an 18 cm zirconium ceramic knife blade (Kyocera, A-Fine GmbH, Hamburg, Germany) on a polyethylene board (Contacto Bänder GmbH, Erkrath, Germany) the entire organ. Afterwards, each minced organ was placed in a sterile plastic laboratory beaker (VitaLab GmbH, Pinneberg, Germany) and homogenized utilizing an Ultra Turrax® T 25 (IKA, Staufen im Breisgau, Germany). In order to reduce cross-contamination each organ was homogenized by using a new beaker and dispersing tool (S 25 D – 14 G – KS, IKA, Staufen im Breisgau, Germany). To avoid overheating of the tissue and to improve the homogenization process for each organ, approximately 4 °C NaCl 0.9 % solution was also added. All homogenate samples, as well as biopsies were weight and stored native at -80 °C.

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