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Scalable SHANEL Tissue Labelling and Clearing for Intact Human Organs

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Abstract

Advances in tissue labelling and clearing methods include improvement of tissue transparency, better preservation of fluorescent signal, compatibility of immunostaining and feasibility for large sample volume. However, existing methods share the common limitation that they can only be applied to human tissue slices, thus rendering intact human organs transparent remains a challenge. In this work, we describe experimental details of the Small-micelle-mediated Human orgAN Efficient clearing and Labelling (SHANEL) pipeline, that can be applied for cellular mapping of intact human organs. We have successfully cleared multiple human organs including kidney, pancreas, heart, lung, spleen and brain, as well as hard tissue like skull. This protocol illustrates the complete process of labelling and clearing whole human organs as well as handling corresponding large image datasets within weeks to months based on the organ types and sizes.

Introduction

Three-dimensional (3D) mapping of the human organs at cellular resolution and generating reference maps of organs across ages or diseases represent the common perspectives of diverse consortiums including HuBMAP (Human Biomolecular Atlas Program)[1,2], Human Cell Atlas[3], Human Tumour Atlas[4], and LungMap[5]. Traditional histological techniques of slicing, staining, imaging and 3D reconstruction of cellular details of biological tissue pieces would receive feasibility and scalability challenges when dealing with big-sized human organs. For example, mapping one intact adult human brain took years of tedious work, not mentioning the mechanical distortion and accidental loss of sections during the lengthy process[6]. While improvements are being constantly developed in the apparatus and iterative image analysis

of multimodality imaging of human organs using PET/MRI, these modalities are still limited by low resolution and the lack of an ability to probe cellular and molecular parameters.

Recently, extensive biomedical research including in neuroscience[7-12], development[13], and cancer[14,15] significantly benefited from the optical tissue labelling and clearing methods that bypass major problems of histology. Especially, clearing and imaging of whole adult mouse bodies opened a holistic examination window into physiological and pathological systems in an unbiased way[9,14]. However, human organs obviously are much larger in size, and contain more complex tissue components due to aging, which are limiting the post-staining methods comparing to months-year old rodent organs. Therefore, whole human organ transparency has been challenging, despite numerous trials on slices[11,16-19]. Hence, we developed a full pipeline of whole human organ labelling, clearing, imaging and 3D map reconstruction at cellular level, which implemented a new tissue labelling and clearing technology termed SHANEL and an advanced volumetric imaging system from commercialized light-sheet fluorescence microscope (LSFM)[20]. This pipeline is easy-to-be-adopted in laboratories, within routine workload, flexible to achieve bona fide scalability (Fig. 1).

Details of SHANEL method

Clarifying the key challenges during the time-consuming endeavour of human organ processing is a prerequisite requirement. Here we delineate the main problems that pose to guide the development of our strategies in Table 1.

Keeping all these questions in mind, we started with introducing the active perfusion system to deliver 0.01 M PBS/heparin and 4% PFA solutions into whole organs through vascular network before the organs harvest if resources are accessible (Fig. 2, Supplementary Fig. 1a). The advantages of this step included washing out remaining blood as much as possible, circulating the vascular system before the forming of clots and faster tissue fixation than passive immersion. The organs were dissected carefully to preserve intact anatomical shapes and connect the main arteries with exogenous tubes for later experiments. Otherwise, human samples could be passively fixed in 4% PFA or 10% formalin buffer to covalently crosslink the proteins. It is worth mentioning that human organ and tissue donation organizations such as IIAM (international institute for the advancement of medicine) are reliable resources, which provide transplantable organs with intact vascular systems and detailed donor information.

Among the hydrophilic reagent-based[21-24], hydrogel-embedding[19,25-27], hydrophobic reagent-based[9,10,13,14] tissue clearing methods, we chose to work with hydrophobic reagents for tissue clearing steps in the SHANEL pipeline. An important advantage of hydrophobic tissue clearing is sample shrinkage, enabling us to accommodate and image large organs using a light sheet fluorescent microscope. Ethanol was employed to get rid of the water inside of human tissue, by increasing serial ethanol concentration step wisely. Dichloromethane was used to extract the remaining lipid and ethanol, after which the tissue was mostly composed of fixed proteins. In the end, the relatively homogenous human tissue became transparent by immersing it into the BABB solution (benzyl benzoate: benzyl alcohol= 2:1, v/v) with a refractive index of 1.56, the same as that of the cross-linked proteins. In general, the hydrophobic reagents cleared organs will shrink ~30% in volume[10,20]. However, traditional commercialized LaVision UltraMicroscope II system (chamber size of 72 x 74 x 35 mm, sample traveling range of 10 x 10 x 10 mm in X,Y,Z) or ZEISS Lightsheet 7 (sample size of

10 x 10 x 20 mm) cannot hold the large cleared human organs, such as an intact eye (size of 30 x 30 x 30 mm). We co-designed together with Miltenyi Biotec and developed a prototype UltraMicroscope (chamber size of 250 x 90 x 70 mm) (Fig. 3). Then Miltenyi Biotec releases commercialized as UltraMicroscope Blaze (chamber size of 129 x 51 x 64 mm, sample travel range of 50 x 24 x 23 mm), fully automated light-sheet microscopes for imaging large cleared samples covering the range from entire mice to most human organs.

Efforts to label and clear human brain pieces by screening with thousands of chemicals[28] or employing extra forces[17,19] have already shown the difficulties regarding to incomplete tissue transparency, time-consuming procedures and limited antibody penetration. Given that aged human tissue is composed of dense and intricate hydrophobic and hydrophilic molecules, we hypothesized that an efficient detergent permeabilization is necessary to render the human tissue accessible to reagents traveling end-to-end through it. Detergents are amphiphilic, possessing both hydrophilic and hydrophobic properties, and forming micelles in solutions that can interact with molecules of the tissue. Traditional detergents of ionic SDS (Sodium dodecyl sulfate) or non-ionic Triton X-100 (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol), carrying typical 'head-to-tail' chemical structures, are inefficient at permeabilizing the sturdy human tissues because their micelle sizes are too big to enter deeply inside of tissue. We identified CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) as a powerful candidate to completely and quickly permeabilize intact human organs, as it forms much smaller micelles with its special 'facial' chemical structure. CHAPS ameliorates the microenvironments of cellular and extracellular matrix of aged human tissue to make the intact human organs permeable and accessible by reagents. To remove the red colour of remaining blood clots, we screened diverse heme-eluting chemical analogues that are compatible with CHAPS. Analogues bearing ethanolamine structures have improved decolorization effects in the presence of CHAPS[20]. Although potential candidates such as N-Butyldiethanolamine[28] and N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine (Quadrol)[24] have been proved to be efficient in decolorizing, but they are at least 2 times more expensive than N-methyldiethanolamine (NMDEA) (~30 €/ liter). Considering the intact human organs consume a large amount of decolorization chemical agents, we decided to use a NMDEA and CHAPS mixture to achieve the permeabilization and decolorization to reduce the cost. Still, the volume of the reagents consumed in a single step of handling an intact human brain was around 5-6 L, and the total cost of all chemicals was about 3200 Euro[20].

Small molecular dyes (<1-2 kDa) or large molecular antibodies (~150 kDa) provide fluorescent contrast of signal-to-background to identify specific structures of human tissue. DNA or RNA chemical probes show high binding specificities and affinities to the nucleic acid of cells across a wide range of fluorescent spectra (e.g. DAPI, Hoechst in blue-green range; JO-PRO-1, PI in green-red range; TO-PRO3, SIR in red range)[29]. Furthermore, chemical fluorochromes conjugated primary or secondary antibodies cannot penetrate and label more than 1 cm deep into the adult human organ due to their big sizes. Hence, we introduced chemical pre-treatments to loosen the cellular and extracellular matrix. First, a solvent mixture of dichloromethane/methanol (2:1, v/v) was used to extract the hydrophobic lipids inside the tissue which would repeal the free movement of hydrophilic labelling reagents dissolved in buffer[30]. Second, the tissue was subjected to acetic acid for partial hydrolysis of intertwined collagen by cleavage of the non-covalent intra- and inter-molecular bonds[31]. This process maintains the collagen chains intact, but the cross-links are cleaved. Third, guanidine hydrochloride buffer was employed to extract the proteoglycans of tissue in

dissociative conditions[32]. After these chemical extraction steps, the spatial interval of tissue matrix became accessible to both small molecular dyes and large molecular antibodies at a depth range centimetres. Cell nuclei in intact human pancreas were perfused and labelled with PI (Fig. 4). Vasculature in multiple human organs was labelled with dextran solution (Fig. 5). SHANEL is also compatible with passive incubation of dye and antibodies to stain PFA fixed human tissue up to centimetres size range (Fig. 6). As we have previously demonstrated, 1.5 cm cubic human brain pieces were successfully labelled with primary antibody and secondary antibody for cellular (e.g. Iba1) and molecular (e.g. tyrosine hydroxylase) structures[20]. Eventually, antibody conjugated with large protein dyes (e.g. phycoerythrin) also fully labelled more than 1 cm cubic human kidney and lung pieces to present cytoplasmic (e.g. Catalase), extracellular (e.g. Collagen IV),and membranous (e.g. cytokeratin 19) structures of tissue (Supplementary Fig. 2 and Table 2).

With the prototype or commercial UltraMicroscope Blaze, it is possible to scan the intact human eye, kidney, thyroid and pancreas by mosaic imaging. The scanning time depends on the size of the sample, the overlapping percentage of the mosaic, the number of scanning channels and the settings of the Z step (Supplementary Fig. 3). Correspondingly, terabytes of large data can be generated from a single organ. Software such as Fiji, Arivis, Imaris and Photoshop can be used to handle the large data for 3D reconstruction and movie generation with a standard lab workstation (e.g., > 256 gigabytes of RAM and terabytes of storage space) (Supplementary Fig. 4-6). However, quantitative analysis of such large data can be difficult and imprecise using such software, which in general relies on simple strategies such as filter-based normalization, thresholding or watershed algorithms. Recently, deep leaning approaches[14,20,33] have shown superior performances in quantification of large-scale data in terms of processing accuracy and speed. It is anticipated that 3D human organ mapping could be greatly advanced with a full exploitation of combing tissue clearing and imaging with deep learning technologies.

Adaptive applications of SHANEL

Mammalian skeletal bones shelter diverse special physiological dynamics and functions across the whole body system. An expanding exploration of deep tissue labelling, clearing and imaging of bones using SHANEL would greatly benefit to investigate the 3D geometric features of bone volume and cells. In addition to the soft tissues such as bone marrow, bones contain hard mineral-dense regions that are deposited with calcium-bearing hydroxyapatite crystals in collagen matrix. The calcium content considerably induces optical scattering of bone[34]. EDTA has been demonstrated as an efficient decalcification reagent in previous studies[35,36]. Similarly, Tainaka et al developed a carbonated hydroxyapatite-based screening system to identify potent decalcification chemicals compatible with the tissue clearing and found EDTA combining with imidazole showed superior effects[28]. Hence, we conducted decalcification of bones using 20% EDTA at 37ºC before SHANEL tissue labelling and clearing, as shown with examples of human skull pieces and pulvinar soft tissue inside the joint cartilage surface surrounded by bones (Fig. 7). Human bone samples are much thicker and harder than mouse ones, which would take much longer time to achieve the desired softness, in the range of weeks to months. There are alternative decalcification reagents composed by strong, mild or weak acids (e.g. nitric acid, formic acid, hydrochloric acid, chromic acid etc.). It is claimed that 5% nitric acid is an option for rapid decalcification while yielding acceptable tissue integrity and antigenicity[37,38].

SHANEL tissue clearing methods have been proven to be applicable to other mammalian species such as pig brain, pig pancreas and be compatible with vDISCO immunostaining[20]. This would enable imaging large mammalian organs that are expressing fluorescent proteins such as GFP, YFP, mCherry, and tdTomato. Since fluorescent protein labeling of cells are widely used including in zebrafish, rat, mouse, pig, and macaque, SHANEL can readily be adopted to clear and image diverse organisms. In such a case, the organs are first, actively perfused or passively incubated with the mixture of CHAPS and NMDEA to permeabilize and decolorize tissue. Afterwards, vDISCO immuostaining process is used via perfusion/incubation with the chosen nanobody in a solution of 1.5 vol% goat serum, 0.5 vol% Triton X-100, 0.5 mM of methyl-β-cyclodextrin, 0.2 wt/vol% trans-1-acetyl-4-hydroxy-L-proline and 0.05 wt/vol% sodium azide in PBS. Finally, the organs become ready to clear by SHANEL reagents. It is anticipated that after CHAPS and NMDEA treatment, nanobody/antibody immunolabeling and SHANEL tissue clearing could be applied to diverse mammalian species to investigate broad biological questions. In cases where antibody-based tissue labelling fails due to its size-limited inability to cross through whole organs, ten times smaller nanobodies could provide a more viable alternative.

Although the SHANEL tissue labelling and clearing method is developed for intact human organs, it also works for small tissue pieces, for example on human biopsies. In general, biopsy samples are small in size and suitable for the application of the passive SHANEL labelling and clearing method.

Comparison with other methods

In the last years, researchers reported their works of labelling and clearing of human organ pieces by diverse methods including CLARITY[17,39], OPTIClear[16], MASH[18], CUBIC[28], SWITCH[40], SHIELD[11] and ELAST[19]. CLARITY and OPTIClear took months to clear fixed human brain tissue pieces (<5 mm thickness). MASH explored the small-molecule fluorescent dye labelling and clearing of human brain cortex (< 5 mm thickness) by modifying the iDISCO protocol. Other methods were applicable to 3D imaging of human myocardial tissue[41], lymph node and lung pieces (< $1cm^3$)[42]. SWITCH allowed multiple rounds of antibody labelling in 100 um thick human brain section. Based on SWITCH method, SHIELD used tissue transformation strategy to stabilize 2 mm thick human brain slice via intramolecular epoxide linkages to prevent degradation. ELAST enabled human brain tissue (< 5 mm thickness) antibody labelling and clearing by mechanically stretching tissue-hydrogel hybrids. The key step of the SHANEL technology relied on the permeabilization and decolorization by CHAPS/NMDEA solution.

For the first time, SHANEL technology achieved the labelling and clearing of intact adult mammalian organs of centimetres sizes, including human brain, pig brain, pig pancreas, human kidney, human thyroid[20], human heart, human pancreas, human lung and spleen (Fig. 5). The whole process can be conducted by passive incubation or active perfusion, depending on the availability of main vessels for external connection to commercialized pumps. This could scale up the process to numbers of organs, with simple set-up in standard labs, and no special expertise or training required to implement. It is preferred to perform active perfusion for large adult organs, in order to speed up the process to a reasonable period (e.g. 1.5 months for human kidney, 4 months for human brain). The timeline for each step varies case by case depending on the specific organs. Even a pair of kidneys from the same donor could be different in size, hardness, blood clots and pigments accumulation. The

organs should be assured to completely fulfil the target of each step of permeabilization and decolorization, delipidation, cellular and extracellular matrix loosening and labelling, dehydration and refractive index matching.

Experimental design

SHANEL provides a flexible platform for diverse human organ resources to achieve whole or partial organ labelling, clearing, and imaging at a cellular level (Fig.1, Supplementary Fig. 7). The whole pipeline is focused on the main steps: (i) preparation of fixed organs according to the donor (steps 1-2); (ii) sample pre-treatment, including optional steps such as decalcification and blood vessel labelling (steps 3-7); (iii) labelling with chemical probes or antibodies (steps 8-14); and (iv) tissue clearing and imaging with light-sheet microscopy (steps 15-17).

Expertise needed to implement the protocol

We would recommend people with knowledge from the biological, medical, or human anatomical background or with previous experience in handling commercial light-sheet microscopes to implement this protocol. With the skills of using Fiji, Imaris, and Arivis software, it will be more quickly to master this method.

Limitations of the current SHANEL pipeline

As most of the tissue clearing methods, the SHANEL tissue labelling and clearing protocol includes the delipidation step, which cannot be compatible with fluorescent lipophilic dyes, such as DiI. One possibility is using modified DiI-analogues, for example CM-DiI, SP-DiI or FM 1-43FX, that could be covalently attached to tissue proteins during aldehyde-fixation. These dyes would adhere to the cellular membranes and proteins, even after lipid clearing[43].

Another issue that we have to take into consideration is the fact that blood clots and lipofuscin cause strong autofluorescence in human samples. The accumulation of lipofuscin is associated with the process of ageing[44]. Its presence would complicate the analyses of specific fluorescence labelling. It has been reported to reduce or eliminate autofluorescence, without adversely affecting the targeted labels, by chemical treatment[45-47] (e.g. $CuSO_4$, Sudan Black B, $NaBH_4$) or photobleaching[19]. We found that $CuSO_4$ was greatly efficient in reducing the lipofusin autofluorescence whilst being compatible with labelling. However, due to the natural blue color of $CuSO_4$ solution, the sample color could be light blue after treatment depending on the concentration of $CuSO_4$ solution, which would partially absorb the laser light energy used during imaging. Therefore, there is a compromise of fluorescence signal-to-background ratio when decreasing the autofluorescence signal. In the future, it would be valuable to identify a colorless, efficient chemical to remove autofluorescence in the SHANEL protocol.

Furthermore, an additional necessary step is the validation of new antibodies, nanobodies or dyes before their application within large organs. Human organs have to be pre-treated with diverse chemicals to loosen the extracellular matrix to allow the antibody or nanobody travel inside. These chemicals would potentially affect the binding of antibody/nanobody with target antigen epitopes of human samples. Nevertheless, positive staining after SHANEL method indicates that the antibody target is well-preserved and specific when comparing the result with previous traditional immunostaining paper. Abundantly commercialized antibodies/nanobodies often require rigorous in-house validation. A well-accepted way of

quickly selecting an antibody compatible, sensitive, and specific with SHANEL, our experience is to choose a research antibody that has been listed in the top-cited ranks in a searchable antibody database[48]. Commonly, human samples firstly go through all pre-treatment steps of CHAPS/NMDEA, delipidation and ECM loosening in SHANEL, then samples were subjected to traditional slicing technique (e.g. cryostat) to generate lots of thin sections (e.g. 10-50 μm). With these sections, the product lot, optimized working concentration and staining conditions were identified. An antibody provides a good signal in tissues sections, which always indicates it has a good chance of being compatible with SHANEL. As mentioned before, confirmed labelling reagents in the lab were summarized in Table 2.

Although the SHANEL tissue labelling and clearing technology is capable to turn human organs as large as the intact brain transparent, current light-sheet fluorescent microscopy have a limited capacity to image all big human organs. In addition, especially for volumetric imaging of whole organs with long-working distance objectives, the current commercial light-sheet microscopes face the problem of poor resolutions in Z, which lead to inevitable non-isotropic volumetric images. In general, whole organ imaging reconstruction data got less influence of non-isotropic volumetric images by quantitative analysis of mesoscale structure. Light-sheet microscope such as MesoSPIM is integrated with a moving-waist beam and can improve the resolution, especially in the Z dimension[49]. The SHANEL method didn't include eliminating the signal from the antibodies stained, and we didn't try the multiple rounds of antibodies relabeling. Efforts on optimized SHANEL method for multiple round labelling of antibodies will also be made.

Materials

Human organ materials

All human organs were taken from different human body donors. All donors gave their informed and written consent to explore their cadavers for research and educational purposes, when still alive and well. The signed consents are kept at the Anatomy Institute, University of Leipzig, Germany. Institutional approval was obtained in accordance to the Saxonian Death and Funeral Act of 1994. The signed body donor consents are available on request.

- Human kidney, pancreas, pulvinar and human brain with skull were dissected from an 88-year-old female donor.
- Human heart, lung, spleen and pancreas for vessel labelling were from a 79-year-old female donor.
- Brain slices for immunolabeling were from an 86-year-old female. Lung and kidney tissue for immunolabeling were from a 97-year-old female donor.

Reagents

- 1x Phosphate-buffered Saline (PBS) (diluted from 0.1 M stock solution, Apotheke Klinikum der Universität Munchen, cat. no. P32799)
- Deionized water (diH_2O, in house purification system)
- 4% PFA in 1x PBS solution (4 wt/vol%; pH 7.4, Morphisto, cat. no. 11762.01000) **!CAUTION** Toxic reagent. **▲CRITICAL** store at 4°C and do not use it when expired.
- Heparin (Braun, 25000 IE/5ml)
- Tetramethylrhodamine isothiocyanate–Dextran (mw 500000, Sigma-Aldrich, cat. no.52194)
- p-maleimidophenyl isocyanate (PMPI) (ThermoFisher, cat. no. 28100) **!CAUTION** Eye and skin irritation.
- Dithiothreitol (DTT) (Sigma-Aldrich, cat. no.43815) **!CAUTION** Eye, skin and respiratory irritation.
- Propidium Iodide (PI) (ThermoFisher, cat. no. P1304MP)
- 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Carl Roth, cat. no.1479.4) **!CAUTION** Light powder, avoid inhalation. **▲CRITICAL** store at 4°C and do not use it when expired.
- N-methyldiethanolamine (NMDEA) (Sigma-Aldrich, cat. no.471828) **!CAUTION** Eye irritation.
- Guanidine hydrochloride (Carl Roth, cat. no. 6069.3) **!CAUTION** Skin and eye irritation.
- Acetic acid (Carl Roth, cat. no. T179.1) **!CAUTION** Danger of flammable and corrosive. Skin and eye damage.
- Sodium acetate (Sigma-Aldrich, cat. no. S2889) **▲CRITICAL** Storage separated from strong acids and strong oxidants.
- Triton X-100 (PanReac Applichem, cat. no. A4975,1000) **!CAUTION** Danger of corrosive, irritant and environmental hazard. Skin and eye damage.
- 2-Hydroxypropyl-beta-cyclodextrin (HPCD, PanReac Applichem, cat. no. A0367,0100)
- DMSO (Carl Roth, cat. no. A994.2) **!CAUTION** skin and eye irritation.
- NaOH (Carl Roth, cat. no. 6771.1) **!CAUTION** Danger of corrosive. Skin and eye damage.

- Ethanol (Merck, cat. no. 10098535000) **!CAUTION** Danger of flammable.
- Methanol (Carl Roth, cat. no. 4627.6) **!CAUTION** Danger of flammable, acute toxic and health hazard. Toxic reagent.
- Dichloromethane (DCM, Carl Roth, cat. no. KK47.1) **!CAUTION** Health hazard. Evaporate.
- BABB (benzyl benzoate:benzyl alcohol = 2:1, Sigma-Aldrich, cat. no. W213802, cat. no. 24122) **!CAUTION** Irritant and environmental hazard.
- Sodium azide (Sigma-Aldrich, cat. no. 71290) **!CAUTION** Danger of acute toxic and environmental hazard.
- Goat serum (GIBCO, cat. no. 16210072)
- Copper sulfate ($CuSO_4$, Carl Roth, cat. no. CP86.1)
- Ammonium chloride (Carl Roth, cat. no. P726.1)

Equipment

- Surgery scissors (FST, cat. no. 14958-11)
- Big Metal Tweezers (FST, cat. no. 11000-20)
- Fine Metal Tweezers (FST, cat. no. 11252-40)
- peristaltic pump (ISMATEC, REGLO Digital MS-4/8 ISM 834)
- PTFE tubing (VWR, 228-0735)
- Reference tubing for the ISMATEC peristaltic pump (ISMATEC, cat. no. SC0026)
- Glass chamber (Omnilab, cat. no. 5163279)
- Glass chamber (LABICAT, cat. no. 40070360, 40070180)
- Shaking rocker (IKA, 2D digital).
- Shaker (IKA, model KS 260 basic)
- 5 ml tubes (Eppendorf, cat. no. 0030 119.401) **▲CRITICAL** highly recommend due to the resistance to clearing solutions
- 15 ml tubes (Thermo Fisher Scientific, cat. no. 339651) **▲CRITICAL** highly recommend due to the resistance to clearing solutions
- 50 ml tubes (Thermo Fisher Scientific, cat. no. 339653) **▲CRITICAL** highly recommend due to the resistance to clearing solutions
- 1 L glass beakers (any, we used the ones from DURAN)
- 1 L glass bottles (any, we used the ones from DURAN)
- 2 L glass bottles (any, we used the ones from DURAN)
- 500 ml glass bottles (any, we used the ones from DURAN)
- PTFE covered magnetic stirring bars (any)
- Hot magnetic stirrer (IKA, model RCT basic B-5000, cat no. 0003810000)
- Aluminium foil (any)
- Plastic wrap (any)
- Disposable underpads (Medimex, cat no. E1911804, or any)
- Syringes of 1 ml, 5 ml, 10 ml, 50 ml (any)
- Tapes, black and transparent ones (any)
- Parafilm (Bemis, cat. no. PM-992)
- pH meter (WTW, model pH7110)
- Shaking rocker (IKA, model 2D digital)
- Incubator (Memmert, model UN160), although any incubator that reaches keeps 37°C with a shaker inside is fine

- Fume hood

Imaging systems

- Computer equipped with light-sheet microscope (Processors: Intel Core i7-7800X CPU @ 3.50GHz; graphic card: NVIDIA Quadro K420; random access memory: 32GB; solid state drive: Samsung SSD 860 PRO 1TB; hard disk drive: Seagate Enterprise Capacity SATA 12TB (ST12000NM0007); operation system: Windows 10 Pro, 64 bit.)

Miltenyi Biotec UltraMicroscope II light-sheet microscope coupled with:

- SuperK EXTREME/FIANIUM supercontinuum white light laser (NKT Photonics, model SuperK EXTREME EXW-12)
- Andor sCMOS camera Neo 5.5 (Andor, mod. no DC-152Q-C00-FI)
- Filter sets: ex 470/40 nm, em 535/50 nm; ex 545/25 nm, em 605/70 nm; ex 560/30 nm, em 609/54 nm; ex 580/25 nm, em 625/30 nm; ex 640/40 nm, em 690/50 nm
- Olympus MVX10 zoom body (zoom range 0.63x-6.3x)
- Olympus revolving zoom body unit (U-TVCAC)
- 1x air objective (Olympus MV PLAPO ×1/0.25 NA, WD = 65 mm)
- 2x immersion objective (Olympus MVPLAPO2XC/0.5 NA, WD = 6 mm)
- 4x immersion objective (Olympus XLFLUOR ×4 corrected/0.28 NA, WD = 10 mm)
- 12x immersion objective (Lavision MI PLAN 12x /0.53NA, WD = 10mm)
- 20x immersion objective (Zeiss ×20 Clr Plan-Neofluar/0.1 NA, WD = 5.6 mm)

Miltenyi Biotec prototype UltraMicroscope light-sheet microscope for large samples coupled with:

- LASOS Multi Color System Series for the laser box (LASOS, model MCS5 F2-01)
- Andor sCMOS camera Zyla 5.5 (Andor, mod. no ZYLA-4.2P-CL10)
- Filter sets: ex 488 nm, em 525/50 nm; ex 561 nm, em 595/40 nm; ex 640 nm, em 680/30 nm; ex 785 nm, em 845/55 nm
- Olympus revolving zoom body that can switch between 1x and 2x with an autofocus-Cube AFC-UM2-UBG
- 1.1x objective (LaVision MI PLAN x1.1/0.1 NA, WD = 17 mm)

Zeiss LSM 880 inverted laser-scanning confocal microscope coupled with

- 25x water-immersion objective (Leica, x25/0.95 NA, WD = 2.5 mm) mounted with a custom mounting thread.

▲**CRITICAL** Check the compatibility of the microscope and objectives of different brands with the vendors.

Computer and Data processing and analysis tools

- Computer (HP Z840 workstation; Windows 10 Pro, 64 bit; CPU: Intel Xeon E5-2640 v3; Installed memory: 256GB; GPU: NVIDIA Quadro M5000)
- Fiji (ImageJ2, https://fiji.sc/) for stitching original mosaic tiffs from light-sheet microscope

- Total Commander (v. 8.52a x64, https://www.ghisler.com/) for rename the file names of stitched tiffs
- ImageMagick (v. 7.0.5-4, https://imagemagick.org/script/index.php) for Lempel-Ziv-Welch (LZW) TIFF compression
- Vision4D (v.3.0.1 ×64, Arivis) for fusing intact organs from multiple scans
- Arivis converter (v.2.12.6, Arivis)
- Imaris (Bitplane) for visualization of 3D images
- Photoshop CS6 (v. 13.0, Adobe)

Reagent setup

Dextran vessel labelling solution

Tetramethylrhodamine isothiocyanate–Dextran is a commonly used dye to trace vessels *in vivo*. To chemically fix dextran inside the vessels, we used p-maleimidophenyl isocyanate (PMPI) and DL-dithiothreitol (DTT) to crosslink the hydroxyl group of dextran to the surround sulfhydryl-containing proteins. The working solution of dextran is containing 5 mg/ml tetramethylrhodamine isothiocyabate-dextran, 0.4 mM PMPI and 1 mM DTT in PBS. For example, as shown in the following table, each reagent is prepared separately. 1) dissolve 8.6 mg PMPI in 2 ml DMSO; 2) dissolve 15.4 mg DTT in 5 ml PBS; 3) dissolve 0.5 g dextran in 93 ml PBS. Mixing all solutions together before the perfusion. (See Table 3)

▲**CRITICAL** Prepare fresh solutions before starting the experiment. **!CAUTION** Prepare the solutions in hood and wear mask.

EDTA solution

EDTA decalcification solution is prepared with 20% (w/v) EDTA in diH_2O, adjusting the PH to 8.0-8.5 using NaOH or HCl. For example, in a 2L bottle with stirring bar, dissolve NaOH continuously to keep 1.5 L diH_2O alkaline, then slowly add 400 g EDTA powder. Replenish diH_2O, more NaOH or HCl until reaching 2L with PH of 8.0-8.5. **!CAUTION** If EDTA precipitation happens, adding more NaOH until the solution becomes transparent.

CHAPS/NMDEA solution

CHAPS/NMDEA mixture is composed of 10% (wt/v) CHAPS and 25% (wt/v) NMDEA (N-methyldiethanolamine) in diH_2O. For example, in a 2 L bottle, mix 200 g of CHAPS and 500 g of NMDEA in diH_2O with a stirrer at room temperature (22-25 ºC) and set the final volume to 2 L. The reagent could be stored at 4 ºC for several months. **!CAUTION** CHAPS is a light powder, it is suggested to wear mask to avoid inhalation during process.

Acetic Acid solution

Acetic acid solution is 0.5M in diH_2O. For example, in a 1L bottle, mix 30 mL acetic acid in diH_2O at room temperature (22-25 ºC) and set the final volume to be 1 L. The reagent could be stored at room temperature for several months. **!CAUTION** Prepare the solution in hood and wear mask due to the smell of acetic acid.

Guanidine solution

Guanidine solution is a mixture of 4 M guanidine hydrochloride, 0.05 M sodium acetate and 2% Triton X-100 in PBS, pH=6.0. For example, in a 2 L bottle, mix 764.2 g guanidine hydrochloride, 8.2 g sodium acetate and 40 g Triton X-100 in PBS with a stirrer at room temperature and set the final volume to 2 L. Adjust the PH to 6.0 with 4M NaOH solution and

30% hydrochloric acid solution. The reagent could be stored at room temperature for several months.

Dehydration and rehydration solutions

A series of dehydration solutions are prepared by mixing ethanol with diH_2O for 50%, 70%, 100% (v/v). It is suggested to prepare the solutions in fresh before using.

Delipidation solutions

The delipidation solution could be DCM/MeOH mixture or pure DCM solution. For tissue pre-treatment before labelling, it is mixture of 2 volume of DCM and 1 volume of MeOH. For tissue clearing after labelling, pure DCM is used. **!CAUTION** DCM is health hazard and highly volatile. It should be handled in a fume hood by wearing safety goggles and double layer nitrile gloves to avoid inhalation and contact with skin/eyes. **▲CRITICAL** Prepare fresh solution before using it.

Sodium Azide stock solution

The concentration of sodium azide stock solution is 0.05% (wt/v) dissolving into 1x PBS. This solution can be stored at 4 °C for several months. **!CAUTION** Avoid contact with skin and eyes.

Blocking buffer for antibody staining

The blocking buffer contains 0.2% (v/v) Triton X-100, 10% (v/v) DMSO and 10% (v/v) goat serum in 1x PBS. For example, in a 500 ml bottle, mix 1 ml Triton X-100, 50 ml DMSO and 50 ml goat serum in PBS with a stirrer at room temperature. **▲CRITICAL** Prepare fresh solution before using it. The pre-treated tissue samples could be stored in blocking buffer at 4 °C if adding 0.01% (w/v) sodium azide for several weeks before antibody staining.

Antibody incubation buffer

The antibody incubation buffer could be composed by 3% (v/v) goat serum, 3% (v/v) DMSO, 0.2% (v/v) Tween-20 and 10 mg/L Heparin in 1x PBS or by 10% (w/v) HPCD, 3% (v/v) goat serum and 10 mg/L Heparin if the antibody water-solubility is poor. **▲CRITICAL** Prepare fresh solution before using it.

Antibody washing buffer

The antibody washing buffer is prepared by 0.2% (v/v) Tween-20 and 10 mg/L Heparin in 1x PBS. This solution can be stored at 4 °C for several months.

Copper sulphate ($CuSO_4$) solution

The copper sulfate solution can be used to eliminate tissue autofluorescence. Dissolve 1.6 g $CuSO_4$ and 2.67 g ammonium chloride in diH_2O, adjust pH to 5.0 and set the final volume to 1 L.

RI matching solution

BABB is the RI matching solution, which is composed by benzyl alcohol and benzyl benzoate with a ratio of 1:2 in volume. BABB solution should be prepared and stored within glass container at room temperature in a fume hood. **!CAUTION** Benzyl benzoate is harmful if swallowed, and cause skin and eye irritation. Benzyl alcohol is harmful is swallowed and inhaled. In hence, handling must be operated by wearing safety goggles, nitrile gloves (preferably double layer) and lab coats.

Equipment setup

Pump system

In active perfusion system, the setting-up are constituted by an Ismatec peristaltic pump and connected tubes. This pump allows four channels as needed (Fig. 2, Supplementary Fig. 1).

Procedure

Preparation of fixed human organs ● Timing 1-2 d

1. Human organs perfusion with PBS/heparin and PFA fixation.
    i. According to the anatomy characteristic of the human organs, use diameter size-matched plastic tubes stitching in the opened arteries and veins. **!CAUTION** Identify any risk factors for acute transmission before human organ handling.
    ii. At room temperature, using a gravitation irrigator system sets up the irrigator height as 0.5m to perfuse the human organs. Marker the start level of PBS/heparin (2.5U heparin per milliliter) solution of the irrigator and perfuse via the plastic tubes with PBS/heparin solution for 3-5 hours until the blood is flushed out with the sign of specimens changed their color to bright. It takes 50 liters of such PBS/heparin solution for a heart-lung block and 20 liters for smaller organs such as kidneys, spleens, or brains.
    iii. Switch the PBS/heparin solution and continue to perfuse with 4% PFA solution for 15 minutes. Control the perfusion speed by setting up the irrigator height as 0.5m. It takes 20 liters per lung/hearth block and 10 liters for the kidneys, spleens, or brains. **!CAUTION** If there was no noticeable flow from the human organs, lift the irrigator to increase the perfusion speed (maximal 1.2 m height). A fume hood is necessary when working with formalin solution, which is toxic with a strong, pungent odor.
    iv. Post-fix the human organs with a 10 times volume 4% PFA solution for 7 days. **!CAUTION** The duration of fixation can affect sample integrity. Optimal fixing conditions should be assessed for each organ and tissue to prevent underfixation or overfixation.
2. Wash the PFA-fixed human organ for following experiments.
    If perfusion applicable, under a fume hood, set up the pump perfusion system with connecting tubing on a layer of disposable pads. Put PFA fixed human organs in a proper glass container and actively perfuse with enough PBS at room temperature for at least three times. The speed of pump is 45-46 rpm (160–170 mmHg), and flow rate is 12.5 ml/min. If not applicable, under a fume hood, put a layer of disposable pads on the shaker. Put PFA fixed human organs in a proper glass container or falcons and passively incubate with enough PBS at room temperature at least three times. The speed of shaker is 50 rpm.
    !CAUTION The connect points of tubing can be protected or sealed with parafilm if needed.
    ■ **PAUSE POINT** The fixed human organs could be stored at 4 ºC in PBS with 0.01% (wt/vol) sodium azide.

Organ pre-treatment according to experimental needs ● Timing 1-14 d

3. In this step, we use different methods to treat different kind of human organs. If the organ vasculature is well preserved, we can perfuse it with dextran vessel labelling

solution. EDTA decalcification is needed in case there is bone tissue to be labelled and cleared.

(A) **Vessel labelling with dextran by perfusion (optional)** ● **Timing 1 d** (Figure. 2)

i. Set up the pump perfusion system with connecting tubing on a layer of disposable pads. Put human organ in an empty and clean glass container, then perfuse it with fresh dextran working solution through each of the organ-inserted tubing until the solution is leaking out, which means the full filling of dextran solution. The speed of pump is 45-46 rpm (160–170 mmHg), and flow rate is 12.5 ml/min. The leaking solution can be collected in a container and reused for another round of perfusion labelling.

▲**CRITICAL** Ensure the organ turn red after dextran injection

ii. After completing vessel labelling, wash the surface of human organ with running water to get rid of the unspecific contaminations from the leaking solution. **!CAUTION** If there is extra dye solution coming out from organ, wipe dry with paper to keep the whole organ surface clean from dye solution.

iii. Put the labelled human organ in a plastic bag and seal with tape.

iv. Put the plastic bag with labelled organ in a container and put them in 37 °C incubator overnight.

v. Take the human organ out and put it into enough PBS at room temperature for washing.

? TROUBLESHOOTING

(B) **Decalcification with EDTA (optional)** ● **Timing 7-20 d**

Incubate human organs with bone tissue in 20% (wt/vol) EDTA (PH≈8.0) with 0.01% (wt/vol) sodium azide solutions at 37 ºC. Refresh the solution every 3-5 days until the bone part gets soft, then wash with PBS. **!CAUTION** EDTA crystals could be precipitated if not sealing well.

Permeabilization and decolorization with CHAPS/NMDEA ● **Timing 3-20 d**

4. If perfusion applicable, continue same pump perfusion system with enough CHAPS/NMDEA solutions to cover the organ for days-month at room temperature, refresh the solution when the solution get green or dark green colour for 2-3 times, until the solution does not change colour anymore. The glass chamber is covered with plastic wrap and aluminium foil to avoid the evaporation of water and light illumination under fume hood (Supplementary Fig. 1c-d).

? TROUBLESHOOTING

5. If not applicable with perfusion, continue with the passive incubation on the shaker. Incubate the organs with enough CHAPS/NMDEA solutions to cover the organ for days at 37 °C, refresh the solution when the solution get green or dark green colour for 2-3 times, until the solution does not change colour anymore.

!CAUTION The reference tubing should be displaced back and forth to different sever-sections every 2 days to avoid the laxation or clamminess after long-term non-stopping abrasion. Check the tubing quality carefully time to time, it happened that the perfusion solution leaked from the wound of tubing if there was accumulation of abrasion from continuous pump running, especially during the

evening time without inspection. The disposable pads were helpful and easy for cleaning up when leaking accident happened.

Delipidation ● Timing 7-50 d

6. At room temperature, under the fume hood, if perfusion applicable, continue with same pump perfusion system, if not applicable, continue with the passive incubation on the shaker.

   ? TROUBLESHOOTING

   i. PBS wash twice in 1 day.
   ii. Dehydrate with 50% EtOH/H_2O solution for 1-7 days.
   iii. Dehydrate with 70% EtOH/H_2O solution for 1-7 days.
   iv. Dehydrate with 100% EtOH solution for 1-7 days.
   v. Dehydrate with 100% EtOH solution for 1-7 days, until the organ does not shrink anymore.
   vi. Delipidate with DCM/MeOH (2:1, v/v) solution for 1-7 days, refresh the solution 2-3 times.
   vii. Rehydrate with 100% EtOH solution for 1-7 days.
   viii. Rehydrate with 70% EtOH/H_2O solution for 1-7 days.
   ix. Rehydrate with 50% EtOH/H_2O solution for 1-7 days.
   x. Rehydrate with diH_2O for 1-7 days.

   !CAUTION The glass chamber should be covered with plastic wrap and aluminium foil to avoid the evaporation of solution and light illumination under fume hood. EtOH, MeOH or DCM is highly volatile liquid, especially under the fume hood. The chamber should be carefully sealed with several layers of plastic wrap and tapes to prevent the volatility of solutions. Replenish the desired solutions to cover the whole sample if needed during the circulation. DCM could erode some types of tubing (e.g. polystyrene), therefore the recommended tubing is from PTFE (polytetrafluoroethylene) or PP (polypropylene). Displace the reference tubing every 2 days.

ECM loosen ● Timing 2-15 d

7. At room temperature, under the fume hood, if perfusion applicable, continue same pump perfusion system, if not applicable, continue with the passive incubation on the shaker.

   ? TROUBLESHOOTING

   i. Collagen hydrolysis with acetic acid solution for 1-7 days.
   ii. DiH_2O wash twice in 1 day.
   iii. Glycoprotein extraction with guanidine solution for 1-7 days.

   !CAUTION The glass chamber should be covered with plastic wrap and aluminium foil to avoid the evaporation of solution and light illumination under fume hood.

Labelling (optional) ● Timing 7-15 d

If perfusion is applicable, continue same pump perfusion system, if not applicable, continue with the passive incubation on the shaker. The whole organ can be stained with cell nuclei dye of PI or TO-PRO-3 solution in PBS (1:1000 dilution) at room temperature for 2-7 days, the solution should cover the whole organ.

If antibody staining is needed, the organ of interest should be tested for certain antibody in sections first (validated antibody list in Table 2, and non-compatible antibody list in Supplementary Table 1). **!CAUTION** Antibody is macromolecule, if perfusion is not applicable, big organs should be dissected into no more than 1.5 cm thick sections to reach antibody full penetration. Actively pumped antibody solutions to stain intact human organ have not yet been tested, while Nanobodies have been proven to stain intact pig organs in active pumping set up.

8. Sample preparation for antibody/dye/nanobody screening: parts of the targeted tissue could be sliced to 40 μm thin sections on glass slides by Cryostat following the standard protocol or cut into 0.3-1 mm floating slices by vibratome following standard protocol. These sections on glass or floating slices could be used for antibody screening.
9. SHANEL pre-treatment for antibody screening: At room temperature, treat the floating slices with CHAPS/NMEDA solution twice per 12 hours. Wash three times with PBS for 20 min each. Gradually dehydrate in 50%, 70% and 100% EtOH/H_2O, 2 hours per step. Change to DCM/MeOH (2:1, v/v) solution overnight. Rehydrate in 100%, 70%, 50% EtOH/H_2O and diH_2O, 2 hours per step. Treat with acetic acid solution overnight, wash twice for 20 min in diH_2O, treat with guanidine solution for 6 hours and wash twice for 20 min in diH_2O.
10. These floating slices are blocked with blocking buffer at 37 °C for overnight, then incubated with commercialized antibodies in antibody incubation buffer at 37 °C for 2 days.
11. The concentration of antibody should be tested according to the manufactures' suggestions. If needed, repeat the incubation with proper secondary antibody at 37 °C for 2 days. The samples are washed with washing buffer three times for 1 hour at room temperature.
12. Gradually dehydrate in 50%, 70% and 100% EtOH/H_2O, 2 hours per step. Change to DCM for 1 hour and then change to BABB until the slice get transparent.
13. Lastly, the results are examined with confocal microscope to find the best conditions for strong and specific antibody staining.
    ▲CRITICAL We strongly suggest using Alexa dye or Atto dye as conjugated fluorophores of antibody, which is stable and bright in clearing solution.
14. After getting the optimistic staining results from antibody screening, dissect the region of interest from the human organ and treat the tissue slice or block following the timing in Table 4.
    ! OPTIONAL Autofluoresecnt lipofusin accumulated in aged human sample, incubate the slice with $CuSO_4$ solution for 1-7 days before clearing to eliminate tissue autofluorescence.
    ? TROUBLESHOOTING

Clearing ● Timing 7-40 d

15. At room temperature, under the fume hood, if perfusion applicable, continue same pump perfusion system, if not applicable, continue with the passive incubation on the shaker. **? TROUBLESHOOTING**
    i. PBS wash twice.
    ii. Dehydrate with 50% EtOH/H_2O solution for 1-7 days.
    iii. Dehydrate with 70% EtOH/H_2O solution for 1-7 days.
    iv. Dehydrate with 100% EtOH solution for 1-7 days.

v. Dehydrate with 100% EtOH solution for 1-7 days, until the organ does not shrink anymore.

vi. Delipidate with DCM solution for 1-7 days, until the sample sink to bottom.

vii. RI matching with BABB solution for 1-30 days until the organ get completely transparent.

!CAUTION The glass chamber should be covered with plastic wrap and aluminium foil to avoid the evaporation of solution and light illumination under fume hood. EtOH or DCM is highly volatile liquid, especially under the fume hood. The chamber should be carefully sealed with several layers of plastic wrap and tapes to prevent the volatility of solutions. Replenish the desired solutions to cover the whole sample if needed during the circulation. DCM could erode some types of tubing (e.g. polystyrene), accordingly, the recommended tubing is from PTFE (polytetrafluoroethylene) or PP (polypropylene). Displace the reference tubing every 2 days (Supplementary Video 1).

■ **PAUSE POINT** The cleared human organs in BABB solution could be stored at room temperature and covered with aluminium foil for months to years before imaging.

Imaging with light-sheet fluorescent microscopy ● **Timing 1-8 d**

16. Here we use the prototype light-sheet microscope from Miltenyi Biotec to image the human pancreas (Fig. 3). The commercialized UltraMicroscope Blaze can also be applied for high resolution imaging of large human organs, they are controlled by the same software named “Imspector”.

    i. Check the sample in BABB solution if there are bubbles inside of organs. If yes, carefully suck out the bubbles using 1 ml syringe. (Supplementary Video 2)

    ii. On pieces of tissue paper, prepare the clean sample holder, glue, tape and samples, for example, human pancreas.

    iii. Cut three pieces of tape and stick on the top, middle, bottom parts of sample holder.

    iv. Add the glue on the tapes of sample holder. To stick the sample durable, the glue is covering full area of the tapes. **!CAUTION** If the glue is not sufficient, the sample is not stable on the holder and would shift or fall down during the 2-3 days of scanning.

    v. Use tissue paper to absorb BABB solution from one side of sample several times.

    vi. The glue would contact the dry side of sample. Carefully put the sample on the sample holder and try to keep the centre of sample aligning with the holder. One end of sample should be close to one end of the moving range of holder (e.g. top to top).

    vii. Waiting for 30-60 seconds until the glue is getting solid.

    viii. Put the sample holder back to the light-sheet microscope and fix it.

    ix. Check again the sample in BABB solution of chamber if there are bubbles inside of organs. If yes, carefully suck out the bubbles using 1 ml syringe. **!CAUTION** Gently touch the sample when sucking out bubbles, otherwise, the glue bonding would be destroyed mechanically and the sample would shift or fall down.

17. Setting imaging parameters in Imspector software. (Supplementary Fig. 3)

    i. Go to “Settings” → “Hardware” → “Ultra3 Settings” → “Objective” to choose the right objective, for example “LVBT 1x”.

ii. Start the View-settings of software with:

iii. “Measurement Mode” to be “Multi Color Mosaic Acquisition”;
“Devices 1” to be “Ultra3 filter”;
“Devices 2” to be “xyz-Table Z”;
“Devices 3” to be “xyz-Table X”;
“Devices 4” to be “xyz-Table Y”;
“AS” means autosave, to be “abled” in green;
“Split” to be “abled for Devices 1”;
“Channel Setup” to click the targeted scanning channels according to the labelling, “Excitation 561, Emission 595/40” and “Excitation 785, Emission empty” here;
“Sheet NA” to be “0.035”, means “Thickness” to be “21 μm”;
“Sheet Width” to be “100%”;
“Zoom” to be “1.0x”;
“Measurement → Liquid” to be “BABB”;
“Measurement → Mount” to be “None”;
“Exposure Time” to be “90.000-100.000”;
“Gain” to be “16-bit low noise”;
“Step” means the step size in um between each two images in Z axis, for example, “-8.00” is set for human pancreas;
!CAUTION The smaller the step size, the more number of images will be acquired, and the scanning will take longer, but the resolution in XZ and YZ direction will increase.

iv. “LightSheet Selection” to be only “left” or only “right” when checking the signal and position of sample.

v. With one side of laser running, adjust the sample stage in Z direction till the laser only illuminates close to top surface of sample as starting position of scanning. (Supplementary Video 3).
!CAUTION Avoid collision of moving objective with the sample in finding focus step, and the sample should only be illuminated on the surface in the starting position step.

vi. Mount the 1.1x objective and then click the “Focus → down” button in the software until the lens get immersed in BABB solution of chamber, adjust the movement of the objective and gets a clear and bright signal place as focus. Then lower the sample holder until the sample is just lower than the light sheet illumination region, click “Current Position → “Set as Zero” and “Scan Range → Start” to be “0.000”.
? TROUBLESHOOTING

vii. Next lifting up the sample holder, the signal gets blurry when imaging plane gets deeper in the sample. Then Click “Scan Range → End”. Since the light sheet microscopy 1.1x objective working distance is around 17mm in z direction, the maximum imaging depth would be -14 mm.

viii. Adjust “Mosaic → Set Parameters” to find the proper “X Axis, Y Axis” and “Overlap” to cover the scanning volume of samples from edges. In general, for 1.1x objective, “3x8” with 30% overlapping or “4x7” with 33% overlapping would cover the human pancreas or human kidney. For 12x objective, “10x10” with 30-40% overlapping is possible. **!CAUTION** Following image stitching will be less accurate if overlapping value is set too small. When setting the

scanning volume, do NOT touch the travelling limitations of stage in X or Y direction, do NOT touch the objective in Z direction. Otherwise, the software would report error and fail.

ix. Choose “Autosave Settings” to fill in the “path” and “name” for saving, such as “humanpancreas_1x_4x7_33o_90ex_8um_56130_78540_forwardup”;

x. Stop the live “View”, and Click the “Lightsheet Selection” to be “both”;

xi. Start the scanning and keep the room in dark for around 2-3 days.

xii. When the scanning finished, take the sample holder out and cut the tapes to remove the sample from holder. Put the sample in BABB solution and pill off the glue and tape at the same time. Wash the sample holder with 100% EtOH and clean with tissue paper.

xiii. Repeat i-xix, change the position of the sample or flip the sample in step vi to make sure the scanning covers both ends and both sides of sample. For example, human pancreas took four times of scanning to finishing the whole organ imaging covering forward-up, forward-down, backward-up, backward-down.

Data stitching, renaming and compression ● Timing 1-2 d

18. When the first round of scanning is finished, TIFF files are generated from light-sheet microscope in the autosave folder. Create new folders inside this autosave folder with names of C00, C01… for the stitched images, corresponding to the image channels.

i. Open Fiji, click “Plugins” → “Stitching” → “deprecated” → “Stitch Sequence of Grids of Images”.

ii. Correct the “Stitch Image Grid Sequence” with the right information from the scans in terms of “grid size x”, “grid size y”, “grid size z”, “overlap”, “Input directory”, “file names”, “Output directory”, “start x”, “start y”, “start z”, “start i”, and the other settings are accepted as default.
“grid size x” to be the number of “X Axis” in light-sheet microscope scanning.
“grid size y” to be the number of “Y Axis” in light-sheet microscope scanning.
“grid size z” to be “1”.
“overlap” to be the number of “Overlap” in light-sheet microscope scanning.
“Input directory” to be the path of the autosave folder.
“file names”: copy the name of first .tif image from the autosave folder to here and replace Ultra3[00 x 00] to be Ultra3[{yy} x {xx}], Z0000 to be Z{zzzz}.
“Output directory” to be the path of “C00” folder inside the autosave folder.
“Start x” to be “0”.
“Start y” to be “0”.
“Start z” to be “any number small than the total number of images”, in general, we choose a number in the middle.
“Start i” to be “1”.

iii. Click “OK” and wait for the running of stitching.

iv. Open the new stitched image (Number is the setting in “Start z”.) in folder “C00” with Fiji to check if the image quality is OK. **! OPTIONAL** Other alternative algorithms are recommended, for example, TeraStitcher (https://abria.github.io/TeraStitcher/).
? TROUBLESHOOTING

v. With the finishing of first stitching, a file called "TileConfiguration.text.registered" would be generated in the "Input folder", which contains the stitching parameters and coordinates of tiles information.

vi. Rename this file to delete the ".registered" part and to generate a .txt file, "TileConfiguration_(zzz)(2)".

vii. Copy this file into folder "C00" and open it, the "C00_xyz-Table Zxxxx" shows the number as set in the step xiv "Start z". Revise the "Zxxxx" to "Z0000" in all tiles.

viii. Copy this new "TileConfiguration_(zzz)(2)" file to other channel folders and revise the "C00_xyz-Table" in all tiles to responding channel number, for example, "C00" to "C01" in folder "C01"; "C00" to "C02" in folder "C02". Each channel requires its own "TileConfiguration_(zzz)(2)" file with corresponding channel number and saved in the respective folder.

ix. Open the file "Stitching.py" with Fiji, which would run the "TileConfiguration_(zzz)(2)" file to finish all stitching.

x. Click "run", and load the "TileConfiguration_(zzz)(2)" file, fill in the total numbers of scanning images.

xi. Repeat vii-viii for each "TileConfiguration_(zzz)(2)" file in each channel folder.

xii. All stitched images would be separately saved in corresponding channel folders, "C00", "C01"…

xiii. Since the stitched tiff images are named with sequential numbers in each channel folder, we would use "Total Commander" software to rename each of the images. Run the software, on the left panel, open the file of stitched folder "C00", choose all "Manual_StitchedZxxxx" images, "Files" → "Multi-Rename Tool" .
"file name" to be "C00_Z". Then click "[C] Counter".
"Define counter [C]" to be "Start at 0", "Step by 1" and "Digits 4".
Click "Start!" button and the software would rename all "Manual_StitchedZxxxx" images in folder C00 to be "C00_Zxxxx".

xiv. Repeat xiii for other channel folders, for example, "C01" to rename the "Manual_StitchedZxxxx" images in folder C01 to be "C01_Zxxxx".

xv. Transfer all stitched images from different channel folders into one folder, for example, "C00".

xvi. Run "LZW compression" using software "ImageMagick" to compress all stitched images in folder "C00".

xvii. Repeat i-xvi for all different volumetric light-sheet microscopy scans. For example, human pancreas would generate four stitched image folders from four times of scanning.

Arivis fusion ● Timing 1-6 d

19. Use "Arivis Vision 4D" to merge multiple 3D volumes.

i. Open "Arivis SIS Converter" → "Add Files" to load all of the stitched images from different channels in C00 folder. If "Assume same structure for all files?" shows up, click "Yes".

ii. "Import Files" → "Custom import" → "Browse" to choose the output folder, → "File Name" to define the name of Arivis file, for example, "Human pancreas_forward up.sis". ▲**CRITICAL** All stitched images from different channels must be in the same folder.

iii. “more options” → “Target Pixel Type” → “16-Bit integer”.

iv. Click “OK” → “Manual import mapper” → “Selection” → “Pattern matching” → “OK” to load the right information of images about channels and image planes.

v. “Start” to convert all stitched images from different channels into Arivis file. **!CAUTION** The .sis Arivis file could be several Terabytes after fusion, for example, human pancreas has 2.5 Terabytes. It is important to save the .sis files in the local drive with enough capacity. The running of Arivis could fail due to poor connection to network drive.

vi. Repeat i-iv to convert all volumetric scans of sample, for example, human pancreas has four Arivis files, corresponding to forward-up, forward-down, backward-up, backward-down.

vii. Double click the first .sis file to open the Arivis Vision 4D.

viii. “Data” → “Pixel Size” to revise the X, Y, Z number to be the correct size from the light-sheet microscopy scanning settings. For example, 1.1x objective has the pixel size of 6.5/1.1=5.909 μm in X and Y; 4x objective has the pixel size of 6.5/4=1.625 μm in X and Y; 12x objective has the pixel size of 6.5/12=0.542 μm in X and Y. The Z pixel size is the “Step size” parameter. Click “Change Pixel Size”. “Save” the file.

ix. Click the “i” button on the left corner to check the image information with “Image Set” and “Plane”, which should be “16 bit” and “number of total images”.

x. To load the second volumetric scans, “File” → “Import” to choose the second .sis file, → “Import Files” → “more options” → “Import as New Image set” → “Target Pixel Type” to be “16 Bit integer”. **▲CRITICAL** The pixel type should be matched for all loaded volumetric files.

xi. When the second .sis file loading finishes, revise the pixel size following step vii again and save the file.

xii. Click the button on the right corner of presenting as “Vertical Split”. “Navigator” → “Images Set” to show the two volumetric scans separately (one is default file), scroll the 2D images to zoom in and out for better view of data, adjust the color bar of each channel for the brightness and contrast.

xiii. If the two volumetric scans are from different side of sample scanning, “Data” → “Transformation Gallery” → “Available Transformations” → “Flipping” → “Flipping Properties” → “Flip X-Axis” or “Flip Y-Axis” or “Flip Z-Axis” to match the X, Y, Z orientations of both volumes. Click “OK” to run it. “Save” the file again after finishing.

xiv. A new image set would show up in the “Image Set”, open the “default” and “flipped” files with same orientations.

xv. In the overlapping volume, identify at least three landmarks in both of the two image sets by zooming in or out and adjusting the planes. **▲CRITICAL** The landmarks could be the same cell, the same hole, the same vessel structure or the same junctions. The landmarks should cover the overlapping volume as much as possible. For example, the first landmark is labeled from the top-right area in the beginning of Z plane, the second landmark is labeled from the middle-left area in the middle of Z plane and the third landmark is labeled from the bottom-right area in the last of Z plane. So the three landmarks could form triangles in any of the XY, YZ and XZ planes. If possible, identify more

landmarks in each of these areas in case wrong labels happen due to the different scanning angle from the light-sheet.

xvi. Click the icon “Place New Object” → “Marker” to label the same landmarks in both image sets and colorful squares would show up. Click “Show Objects Table” to view the information of markers.

xvii. Click “Data” → “Volume Fusion” → “Image Sets” to choose the right “Base Image Set” and “Moving Image Set”, “Save as” choose “New File”, “Image Set Name” to define the name for new fusion. “Transformation” → “landmark Registration” → “Base Image Set” → “ Add All Objects as Landmarks” by right clicking the set icon. Do the same for “Moving Image Set”. Right click marker to delete the unwanted ones. In the end, three same landmarks are enough. “Scale” to be “10%”. Click “Run” to finish the trials of fast volume fusion for preview. **▲CRITICAL** The sequence of landmarks should be the same in both image sets. The highlighted locations of landmarks would display in the right window.

xviii. If the new fusion is not good, for example, with mismatching, gap or ghosting, try different combinations of three different landmarks to get the best fusion quality in both 2D view and 3D view.

xix. If fusion is good enough, change the “Scale” to be “100%” and run the final fusion to generate the new .sis file with both volume sets.

xx. Click “Show Objects Table” to view the information of markers, → “Im/Export” → “Excel Export” → “Save As” to choose the folder, → “Export” to keep the record of landmarks for both image sets.

xxi. Repeat step vi-xix to fuse each of other parts of volumetric scan into the new fused file. For example, repeat three times of the process to generate the final 3D fusion .sis file for human pancreas.

xxii. “File” → “Export” → “TIFF Exporter” and define the file name in a new folder to generate a series of .tif images. All final fused images from all channels would be saved in the folder.

xxiii. Run “Total Commander” software to rename each of the images. Run the software, on the left panel, open the files in the saved folder, “Mark” → “Select Group” → input “C00” → “OK” to choose all files containing the “C00”.

xxiv. “Files” → “Multi-Rename Tool” → “file name” to be “C00_Z”, Click “[C] Counter”, “Define counter [C]” to be “Start at 0”, “Step by 1” and “Digits 4”. Click “Start!” button and the software would rename all images with “C00” in the saved folder to be “C00_Zxxxx”.

xxv. Repeat xxii-xxiii to rename the fused images for other channels.

xxvi. Run “LZW TIFF 16bit” to compress all fused images in the saved folder.

? TROUBLESHOOTING

Imaris 3D visualization ● Timing ~0.5-2 d

20. Here we only include the basic and simple steps for Imaris 3D visualization. For more functional analysis, please check the Imaris website for user guide.

i. Open “Imaris File Converter” → “Add Files” to load all of the fused images in the saved folder. Define the save path in “Output”.

ii. Click on the loaded files → “Settings” → Select “File names with delimiter”, confirm the format as C“C”_Z“Z”.tif. Then click “Start All”.

iii. Following the save path, an “Imaris Image File” is generated.

iv. Double click to open the Imaris file, “Edit” → “Image Properties” → “Geomertry” → “Voxel Size”. X=6.5/objective, Y=6.5/objective, Z= “z” step size in the imaging setting. For example, if choosing the 1x Objective with a z step of 8 μm, then X=6.5 μm, Y=6.5 μm, Z=8 μm. if choosing the 12x Objective with a z step of 6 μm, then X=0.542 μm, Y=0.542 μm, Z= 6 μm.

v. “Edit” → “Show Display Adjustment” to change the channel colors and adjust the contrast.

vi. “Imaging Processing” → “Camera Function” → “Set The Angle Of The Camera” to set needed 3D views.

vii. “Snapshot” → “Preferences” → “Snapshot” to set the Size, DPI (at least 300), Save as Type (TIFF images), Image Output Directory.

Timing

See Table 4.

Troubleshooting

See Table 5.

Anticipated Results

We described SHANEL in the presented protocol as a scalable tissue labelling and clearing method for a variety of human organs (Supplementary Video 4), including pancreas (Figs. 3-5), kidney, and additional vessel labelling of spleen, heart and lung compared to our original publication (Fig. 5), we also extend the original method to be compatible with human hard tissue with bone (Fig. 7). SHANEL allows imaging of intact human organs at cellular resolution, which paves the way for human organ mapping, and potentially extends our knowledge in human organ anatomy. The technique can be used to study vascular morphology and pathology, as the fine details of capillary can be visualized throughout whole organ (Fig. 5).

We previously used SHANEL to label and quantify the pancreas islets in *INS*-EGFP transgenic pig[20], demonstrating the scalability of this method to study large scale biological samples. SHANEL is also applicable for antibody staining in human tissue or biopsies (Fig. 6), increasing its adaptability in biomedical research.

After using the recently developed LSFM to image the transparent human organs or tissues and to therefore acquire spatial information of vessels and fluorescent protein signal (Fig. 3, Supplementary Fig. 3), the whole data set can be efficiently stitched with an easily operated software (Supplementary Fig. 4). Here, we show an example of how raw images were processed and visualization for the entire 3D fields and cellular states information was rapidly achieved in a simple-to-use software (Supplementary Fig. 5-6). Note that, due to the size of human organs, terabytes of the image data will be generated. Consequently, it would be suggested to combine our methodology with deep learning approaches to achieve accuracy and speed in quantification and further analysis. We summarize a workflow (Supplementary Fig. 7) that will be useful for researchers in order to select critical steps and apply this protocol.

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Author Contributions

A.E. conceived and led the project. S.Z. developed the original SHANEL protocol. H.M., Z.R. and S.Z. performed the experiments and wrote the manuscript. R.C. performed the antibody screening. H.S. and I.B. dissected and provided the human organs. All authors commented on the manuscript text.

Data availability

The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

A.E. and S.Z. filed a patent on SHANEL technologies described in this study.

Additional information

Supplementary information is available at http://discotechnologies.org/SHANEL/.

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Figure Legends

Figure 1. Overview of SHANEL pipeline

SHANEL is composed of seven parts. After human organs and tissue are collected (Step 1), human organs preparation is performed for removing blood and fixation(Step 2). The continuous step is divided into active pumping (Step 3a) and passive incubation(Step 3b), based on whether the organs are intact or not. Samples are then following permeabilization, delipidation, ECM loosening, and chemical probes labelling. According to the antibodies compatible with the SHANEL protocol, the organ of interest can be dissected into less than 1.5cm thick slices for immunolabelling (Step 4). Samples are then dehydrated, delipidated, and matched for refractive index, until transparent (Step 5). Imaging of whole human organs is performed in an UltraMicroscope Blaze LSFM, and tissue slices are captured with an UltraMicroscope II LSFM for high resolution (Step 6). The data is stitched, volumes fused, and rendered in 3D visualization (Step 7).

Figure 2. Vessel labelling with dextran solution by active perfusion

(a) Photo of dissected human kidney with inserted tubing (scale bar: 1 cm). **(b)** The tubing was changed to be PTFE (chemical-resistant, anti-adhesive, biocompatible) tube and tightly fixed by double rope-fastening (red rectangle in **c**). **(d)** The inserted nozzle of PTFE tube was cut with a slope angle to plug-in smoothly. **(e)** A set of connecting tubing contained the black pump reference tube in the middle, two white tubes at both ends, tube connectors and a pipette. The red arrows indicated the flow circulation direction. **(f)** Vessel labelling with dextran solution using pump perfusion system (rotation of the wheel in **g** enables solution flow into kidney, black pump reference tube was fastened by correct set up of cassette shown in **h**). **(i)** Photos of human kidney after dextran labelling. **(j)** The dextran labeled human kidney was sealed in a plastic bag.

Figure 3. Organ mounting and imaging with light-sheet microscopy

(a) Photo of human pancreas before clearing (scale bar: 1 cm). **(b)** Photo of transparent human pancreas after PI cell nuclei labelling and clearing, showing mesenteric artery (white arrow) and lymph nodes (yellow asterisk) (scale bar: 1 cm). **(c)** The mounting of an organ on a sample holder required glue and black tape. The red arrows show the moving range ends of holder in Y direction. **(d)** The sample holder was gummed with four sections of tape and dropped with glue. **(e)** One example of sample position with one end alignment to cover one sample edge. **(f)** Another example of sample position with another end alignment to cover the other sample edge. **(g)** The mounted human pancreas was illuminated by light sheet.

Figure 4. 3D reconstruction of human pancreas

(a) 3D reconstruction of human pancreas imaged by light sheet fluorescence microscopy focusing on the PI labeled cell nuclei (Green) and autofluorescence of 785 nm wavelength (magenta) (scale bar: 1 cm). **(b)** Zoomed 3D reconstruction in figure A showed the vessels and lymph nodes (red rectangular) (scale bars: 1 mm and 200um respectively). **(c)** 3D reconstruction of one example of endocrine portion by high magnification of 12X objective (scale bar: 200 um). **(d)** Diverse islets surrounded by acinar cells could be easily identified in 3D images of endocrine portion showing standard round or oval shape and irregular shapes as well as variable sizes (red asterisk). Also, intralobular ducts could be located near the islets (yellow arrow). (scale bars: 50 um, 50 um and 30um, respectively).

Figure 5. SHANEL of human organs with perfusion of dextran vessel labelling dye
(**a**) Human heart after SHANEL protocol (i), 3D reconstruction (ii) shows blood vessels of coronary artery (red) and myocardium which can be imaged in autofluorescence at 488 nm (AF, green) (scale bars: 1 cm and 2mm, respectively). (**b**) Human kidney after SHANEL (i), structure of glomeruli can be seen in dextran labelled. TO-PRO3 labelled cell nucleus (scale bars: 1 cm and 2mm, respectively). (**c**) One lobe of human lung was cleared and stained with dextran, bronchus can be seen in AF (scale bars: 1 cm and 2mm, respectively). (**d**) Image of human pancreas and vasculature after SHANEL (scale bars: 1 cm and 2mm, respectively). (**e**) Human spleen after SHANEL, fine structure of splenic artery was visualized in Imaris (scale bars: 1 cm and 2mm, respectively).

Figure 6. SHANEL of human tissues by passive incubation
(a) Skull labelled with lectin (yellow) and PI (blue) (i). XY section (ii) and magnified image (iii) (scale bars: 1mm, 1mm and 100um, respectively). (**b**) brain slice labelled with Neurotrace Nissl stain. (scale bars: 2mm and 300um, respectively). (**c**) brain tissue labelled with Iba1 antibody (i). YZ (ii) and XZ (iii) section, magnified image in XY section (iv) (scale bars: 1000 um, 700 um, 500 um and 40 um, respectively). (**d**) human lung tissue labelled with α-SMA antibody (i). YZ (ii) and XZ (iii) section, magnified image in XY section (iv) (scale bars: 700 um, 500 um, 500 um and 150 um, respectively).

Figure 7. Passive incubation, clearing and 3D reconstruction of human pulvinar and skull
(a) Photo of transparent human pulvinar after PI cell nuclei labelling and clearing by passive incubation (scale bar: 1 cm). **(b)** 3D reconstruction of human pulvinar imaged by light-sheet fluorescence microscopy focusing on autofluorescence (AF) of 488 nm wavelength (grey) and PI labeled cell nuclei (Green) (scale bar: 1 cm). **(c)** section view of pulvinar showing the connection between bone tissue and the pulvinar fibrofatty tissue (scale bar: 150 um). **(d)** Human skull bone after PI cell nuclei labelling and clearing (scale bar: 1 cm). **(e)** The human skull imaged by light-sheet and confocal microscopy (Scale bar 500 um and 200 um, respectively).

Supplementary Figure 1. Human brain clearing by perfusion system
(a) The dissection of human brain with CL and CR (left and right carotids, respectively), VL and VR (left and right veterbral arteries, respectively). **(b)** The human brain after dissection and fixation. **(c)** The set up of human brain clearing by perfusion system under fume hood. The human brain was put in a glass container and connected with input and output tubing controlled by peristaltic pump. There were four channels to control the four connecting tubings as indicated in C i. Two output tubings were connected with the carotids arteries as shown in C ii and two output tubings were connected with the vertebral arteries as shown in C iii. The input tubings were protected with gauze to avoid solid impurities entering and blocking the tubings (see C iv). C v showed the decolorization effects of CHAPS/NMDEA according to the blooming of dark green color from the organ. **(d)** The glass container was sealed with several layers of plastic wrap to prevent the volatility of running solutions, especially for EtOH, DCM, MeOH. **(e)** Photo of human brain after SHANEL pre-treatment before clearing. Remaining blood in the vessels (**b**) was decolorized.

Supplementary Figure 2. Passively stained conjugated antibody in centimeter-size human kidney and lung tissue

Collagen IV, catalase and cytokeratin 19 antibody staining (Green) in human kidney (**a**) and lung (**b**). Top panel shows immunofluorescent staining images in confocal microscopy to verify antibody compatibility with SHANEL. Bottom panel shows 3D reconstruction of tissue with antibody labelling in Imaris. (scale bars: 20 um and 500 um, respectively)

Supplementary Figure 3. Settings in imaging software

(a) The user interface of Lavision Imspector software. (i) refer to the setting of objective lens and magnification. **(ii)** The names of the devices should be listed in the correct order. **(iii)** One example for settings of the tiling scan with 4x7 tiles and 33% overlap.

Supplementary Figure 4. Key steps for data stitching, renaming and compression

(a) One example for correcting information of 'Stitching Image Grid Sequence', and a1 highlighted the replacement of red letters in step x about 'file names'. **(b)** The key information of 'TileConfiguration' txt file, b1 highlighted the responding channel number should be corrected and saved in respective separated folder and b2 highlighted the starting Z number should be '0000' for all 'TileConfiguration' files. **(c)** The information of 'Stitching.py'. **(d)** One example for settings of 'Multi-Rename Tool'. **(e)** One example of compressing tiff files with 'LZW TIFF'.

Supplementary Figure 5. Key steps for Arivis fusion of 3D images

(a) One example of flipping one volumetric data of X or Y or Z direction to match with another volumetric data. **(b)** The overview of two volumetric data showing same XYZ directions after flipping. **(c-d)** Examples of identical structural markers from two volumetric data sets for fusion. **(e)** Loading of three key markers for best fusion. **(f)** 3D fused image of pancreas from two volumetric data sets.

Supplementary Figure 6. Key steps for Imaris data loading and visualization

(a) One example of converting stitched image sequence data to the .ims format using Imaris File Converter. (**b**) The settings for file names with delimiter should be changed into "C _ Z.tif". (**c**) 3D image properties including XYZ voxel size and channel color can be set in Imaris for analysis.

Supplementary Figure 7. Overview of SHANEL workflow

This workflow summarizes the main steps after organ collection: experimental setup (yellow), pre-treatments (blue), labelling and clearing (grey) and imaging (red). We provided two ways of handling samples: active perfusion with pump, if the vessels could be connected with external tube, and passive incubation with shaker. Moreover, depending on the organ components, organ size, targeted labelling, and imaging structurers, we outlined the differences in the whole process.

Table 1| Challenges of human organ labelling, clearing and imaging
1. Uncontrollable variables from human organ resources related to the pre-mortem state and post-mortem delay. ***Solution:*** Selecting reliable human organ and tissue donation organizations.
2. Extremely complicated chemical compositions in terms of lipidome complexity, myelin density, lipofuscin accumulation and non-soluble, non-enzymatic collagen changes. ***Solution:*** An efficient detergent permeabilization is necessary to render the human tissue accessible to reagents traveling end-to-end through it.
3. Residue blood clots due to the delay of post-mortem dissection. ***Solution:*** Setting up active perfusion system to deliver 0.01 M PBS/heparin before 4% PFA fixation can reduce the blood remain in the vessel as much as possible.
4. Strong autofluorescence at visible wavelengths (400-700 nm) from lipofuscin. ***Solution:*** chemical treatment (e.g. $CuSO_4$, Sudan Black B, $NaBH_4$) or photobleaching has been reported to reduce autofluorescence. We found that $CuSO_4$ was greatly efficient in reducing the autofluorescence whilst being compatible with labelling.
5. Advanced transgenic or virus tracing techniques for fluorescent labelling not being applicable for human organs. ***Solution:*** Selecting high binding specificities and affinities chemical probes or antibodies for human organs labelling.
6. Increased costs of reagents and dyes due to the size of human organs. ***Solution:*** Using lower price N-methyldiethanolamine (NMDEA) and CHAPS mixture to achieve the permeabilization and decolorization.
7. Volume of large-sized human organs exceeds the limit of volumetric imaging. ***Solution:*** Developing larger chamber size fully automated light-sheet microscopes to cover the range from entire mice to most human organs.
8. Massive imaging data would be generated and need to be stored and analysed. ***Solution:*** Lempel-Ziv-Welch (LZW) TIFF compression and equip with network attached storage.

Table 2| Dyes and antibodies compatible with SHANEL protocol

	Supplier	Cat no.	Dilution
Hoechst	Invitrogen	H3570	1:1000
PI	Invitrogen	P3566	1:1000
TO-PRO-3	Invitrogen	T3605	1:1000
Neuro trace Nissl	Invitrogen	N21483	1:1000
DRAQ5	Thermo	62251	1:1000

	Fisher			
Methoxy-X04	Tocris	4920	100 uM	
Dextran	Sigma	52194	5 mg/ml	
Congo Red	Sigma	C6277	10 uM	
Lycopersicon Esculentum (Tomato) Lectin	Vector	DL1174	1:200	
Lycopersicon Esculentum (Tomato) Lectin	Vector	DL1178	1:200	
Antibodies	Supplier	Cat no.	Dilution	RRID
Tyrosine Hydroxylase	abcam	ab112	1:200	AB_297840
Ionized calcium binding adapter protein 1 (Iba1)	Wako	019-19741	1:500	AB_839504
Neuropeptide Y	abcam	ab30914	1:200	AB_1566510
Myelin basic protein	Atlas	AMAb91064	1:200	AB_2665784
Laminin	Sigma	L9393	1:200	AB_477163
Alpha-smooth muscle actin	Sigma	C6198	1:500	AB_1840528
CD8	abcam	ab237709	1:200	NA
Angiotensin-converting enzyme 2 (ACE2)	Invitrogen	PA5-20039	1:200	AB_11154831
Conjugated antibodies				
Atto647N-conjugated anti-GFP nanobooster	Chromotek	gba647n-100	1:1000	AB_2629215
Collagen IV	Miltenyi	130-122-866	1:100	AB_2857566
Catalase	Miltenyi	130-123-367	1:100	AB_2857591
Cytokeratin 19	Miltenyi	130-125-272	1:100	AB_2857769
Antibodies tested on mouse tissue	Supplier	Cat no.	Dilution	RRID
Peripheral-Type Benzodiazepine Receptor	abcam	ab109497	1:500	AB_10862345
Cleaved Caspase9	CST	9509	1:500	AB_2073476
Caspase 9	abcam	ab184786	1:500	NA
Gama-H2AX	Invitrogen	14-9865-82	1:500	AB_2573048

Table 3| Preparation of dextran working solution

	PMPI	DTT	Dextran	PBS
MW(g/mol)	214.2	154.2	500,000	-
Stock concentration	20 mM	20 mM	-	-
Stock solution	DMSO	PBS	-	-
Weight (mg)	8.6 mg	15.4 mg	500 mg	
Volume of solution	2 ml	5 ml	-	93 ml

Table 4 | Timing

Sample type	Human Kidney	Human Pancreas	Human Spleen	Human heart/lung	Human Pulvinar	Human Brain	Human Skull	Tissue slice/ block
PBS wash	1d	1d	1d	1d	1d	2d	1d	1d
Vessel labelling	1d	1d	1d	1d	-	-	-	-
EDTA	-	-	-	-	7d-10d	-	14-20d	-
CHAPS/NMDEA	10d-14d	5d-7d	5d-7d	10d-14d	5d-7d	20d	5d	3d
PBS	1d	1d	1d	2d	1d	3d	1d	1d
50% EtOH	1d	1d	1d	2d	1d	5d	1d	1d
70% EtOH	2d	1d	1d	2d	1d	7d	1d	1d
100% EtOH	2d	1d	1d	3d	1d	7d	1d	1d
100% EtOH	2d+2d	1d	1d	3d	1d	7d	1d	1d
DCM/MeOH	3d	2d	2d	3d	2d	7d	2d	2d
100% EtOH	1d	1d	1d	2d	1d	4d	1d	1d
70% EtOH	2d	1d	1d	2d	1d	5d	1d	1d
50% EtOH	2d	1d	1d	2d	1d	5d	1d	1d
DiH_2O	1d	1d	1d	2d	1d	3d	8h	8h
Acetic Acid	2d	1d	1d	2d	1d	7d	1d	1d
DiH_2O	8h	8h	8h	2d	8h	3d	8h	8h
Guanidine HCl	1d	1d	1d	2d	1d	7d	1d	1d
PBS	8h	8h	8h	2d	8h	3d	8h	1d
Cell Nuclei Dye	7d	7d	7d	7d	7d	-	2d	-
Antibody Labelling	-	-	-	-	-	-	-	7-14d
PBS	2d	1d	1d	2d	1d	-	1d	1d
50% EtOH	1d	1d	1d	2d	1d	5d	1d	1d
70% EtOH	2d	1d	1d	2d	1d	7d	1d	1d
100% EtOH	2d	1d	1d	2d	1d	7d	1d	1d
100% EtOH	2d	1d	1d	3d	1d	7d	1d	1d
DCM	3d	1d	1d	3d	1d	7d	1d	1d
BABB	3d-	2d-	2d-	5d-	2d-	7d-	2d-	1d-
Total processing time for each organ	~54-60d	~36-38d	~36-38d	~69-73d	~43-48d	~135d	~43-49d	~32-39d

1360 Table 5 | Troubleshooting table

Step	Problem	Possible reason	Solution
3	Undissolved deposits in dextran vessel labelling solution	Not sufficient mixture	Prepare stock solution and mixture step by step
	Difficult to find vessel	Some organs have tube structure similar to vessel. For example, urine tract in kidney and trachea in lung	Confirm the main arteries from anatomy text book, or ask experts for help
	Organ cannot turn red after dextran injection	Blockages in blood vessels	Use higher pressure or a 50ml syringe to inject dextran solution
4, 6, 7, 14	Solution leakage during active pumping	Disconnection or breakage of tubing	Change new tubing to continue pumping
15	Poor organ shrinkage after clearing	Insufficient incubation with ethanol	Increase incubation time with ethanol
17	The samples are not transparent enough and blurry under microscopy	The samples are not dehydrated enough	Make sure the organs dehydrated enough from the sign of no more shrinkage
		The samples are not in sufficient time of BABB	put the sample in BABB until transparent
17	Shade inside the sample	Not sufficient light sheet width	Optimize sufficient light sheet width
		Light absorption in surface by high-cell density region	Try staining with a lower concentration of dye
17	Vessel labelling only in some regions of the organs	The vessel of organ is not intact	Collect the organs carefully and try not to damage it
		Residual blood clot in the sample	Perfuse sample with sufficient PBS and Heparin before organ harvest
17	Signal cannot be detected in the deeper tissue layer	Short wavelength light cannot penetrate to the tissue due to scattering	Use near infrared wavelength or longer wave length chemical probe or secondary antibodies.
17	Staining worked only on the sample surface	Incubation times were too short, or antibody incubation buffer volumes were too small.	To process repeated staining, samples can be gradually rehydrated starting from Step 6 Vii. Also increase incubation times and/or antibody incubation buffer

			volume
17	Sample are not visible during the imaging	Out of focus	Move the stage to find bright halos under objective and set up the right contrast on the computer screen.
17	No positive signal, although the antibody works in IF on tissue sections	Incompatibility with SHANEL protocol	Check antibodies list or prototype with sections pre-treated with SHANEL protocol.
17	Weak or non-detectable antibody signals	Insufficient antibody labelling or incapable antibody for SHANEL protocol	Select the antibody form our verified antibody list or optimize the antibody staining conditions.
17	High autofluorescence	Too long PFA fixation time	Reduce PFA fixation time. Fix the sample in PFA less than four weeks. Change to PBS with sodium azide for long-term storage.
18	Stitching failed with the tiling scans	Sample has movement during imaging	Use enough BABB resistant glue to fix the sample or use a custom made holder to fix the sample.
18	Shade inside the sample	Obstruction, such as bubbles	Remove obstruction, such as using syringe to withdraw bubbles.
18	Data are too large to analyze	Unnecessarily high z-resolution Unnecessarily high overlapping percentage of mosaic	Optimize the Z steps based on the maximum possible z-resolution of the lens. Optimize overlapping percentage
19	Failed with the stitching grids of Images	sample move during scanning	Manual stitching or make sure the sample well fixed in the holder

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Supplementary table 1| Antibodies not compatible with SHANEL protocol

Antibodies	Supplier	Cat no.
APC	Calbiochem	OP80100UG
GFAP	Sigma	G3893
NeuN	Millipore	MAB377
Calretinin	abcam	ab702
MMP13	abcam	ab39012
myelin basic protein	abcam	ab7349
Parallbumin	abcam	ab11427
CD133/1	Miltenyi Biotec	130-115-292
Podoplanin	Miltenyi Biotec	130-121-373
CD71	Miltenyi Biotec	130-124-358
CD90	Miltenyi Biotec	130-124-176
CD38	Miltenyi Biotec	130-123-347
JNK2 (MAPK9)	Miltenyi Biotec	130-122-868
CD39	Miltenyi Biotec	130-125-078
WT1	Miltenyi Biotec	130-125-782
CD13	Miltenyi Biotec	130-125-787
CD272 (BTLA)	Miltenyi Biotec	130-125-966

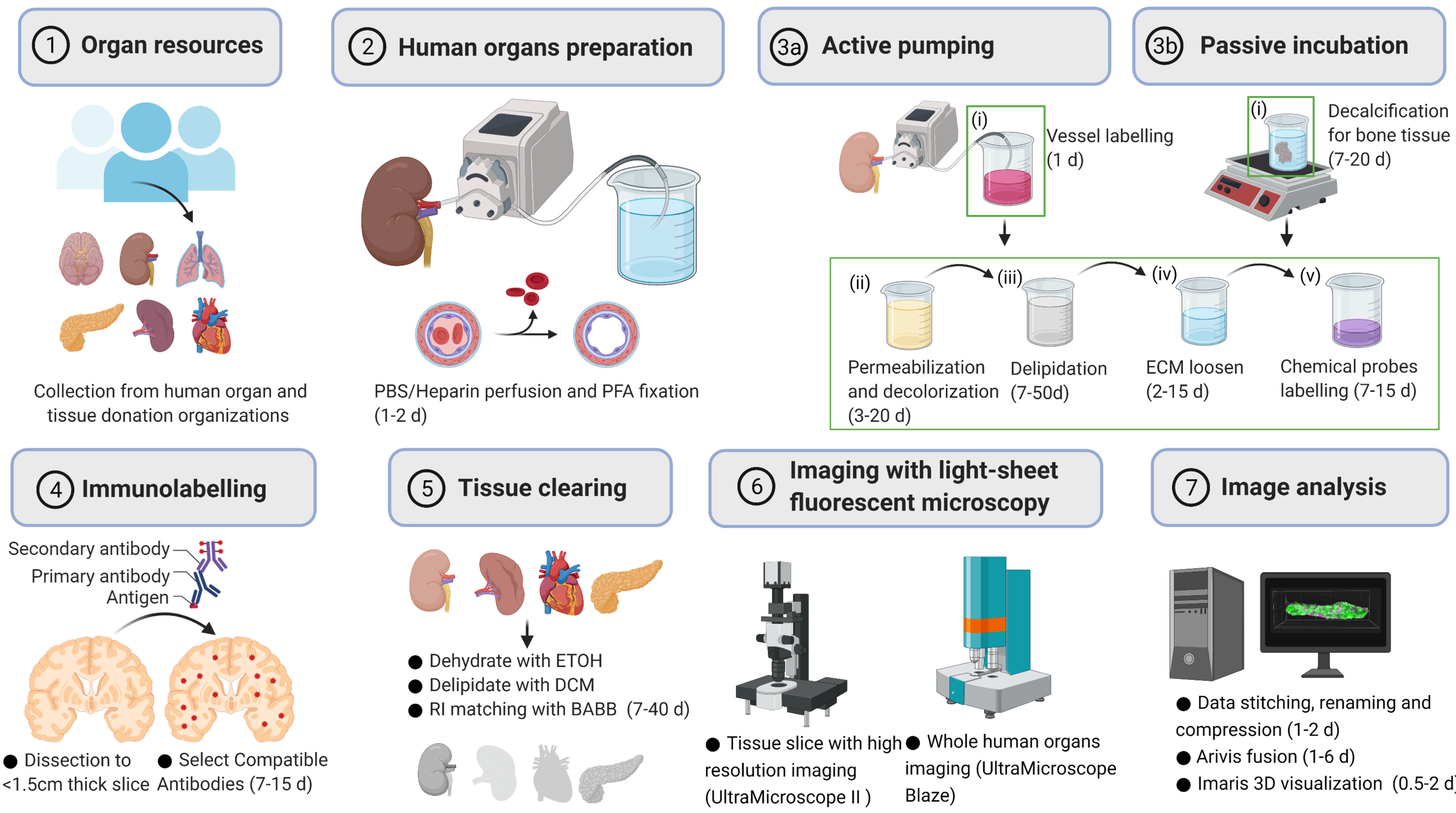


Figure 1. Overview of SHANEL pipeline

SHANEL is composed of seven parts. After human organs and tissue are collected (Step 1), human organs preparation is performed for removing blood and fixation(Step 2). The continuous step is divided into active pumping (Step 3a) and passive incubation(Step 3b), based on whether the organs are intact or not. Samples are then following permeabilization, delipidation, ECM loosening, and chemical probes labelling. According to the antibodies compatible with the SHANEL protocol, the organ of interest can be dissected into less than 1.5cm thick slices for immunolabelling (Step 4). Samples are then dehydrated, delipidated, and matched for refractive index, until transparent (Step 5). Imaging of whole human organs is performed in an UltraMicroscope Blaze LSFM, and tissue slices are captured with an UltraMicroscope II LSFM for high resolution (Step 6). The data is stitched, volumes fused, and rendered in 3D visualization (Step 7).

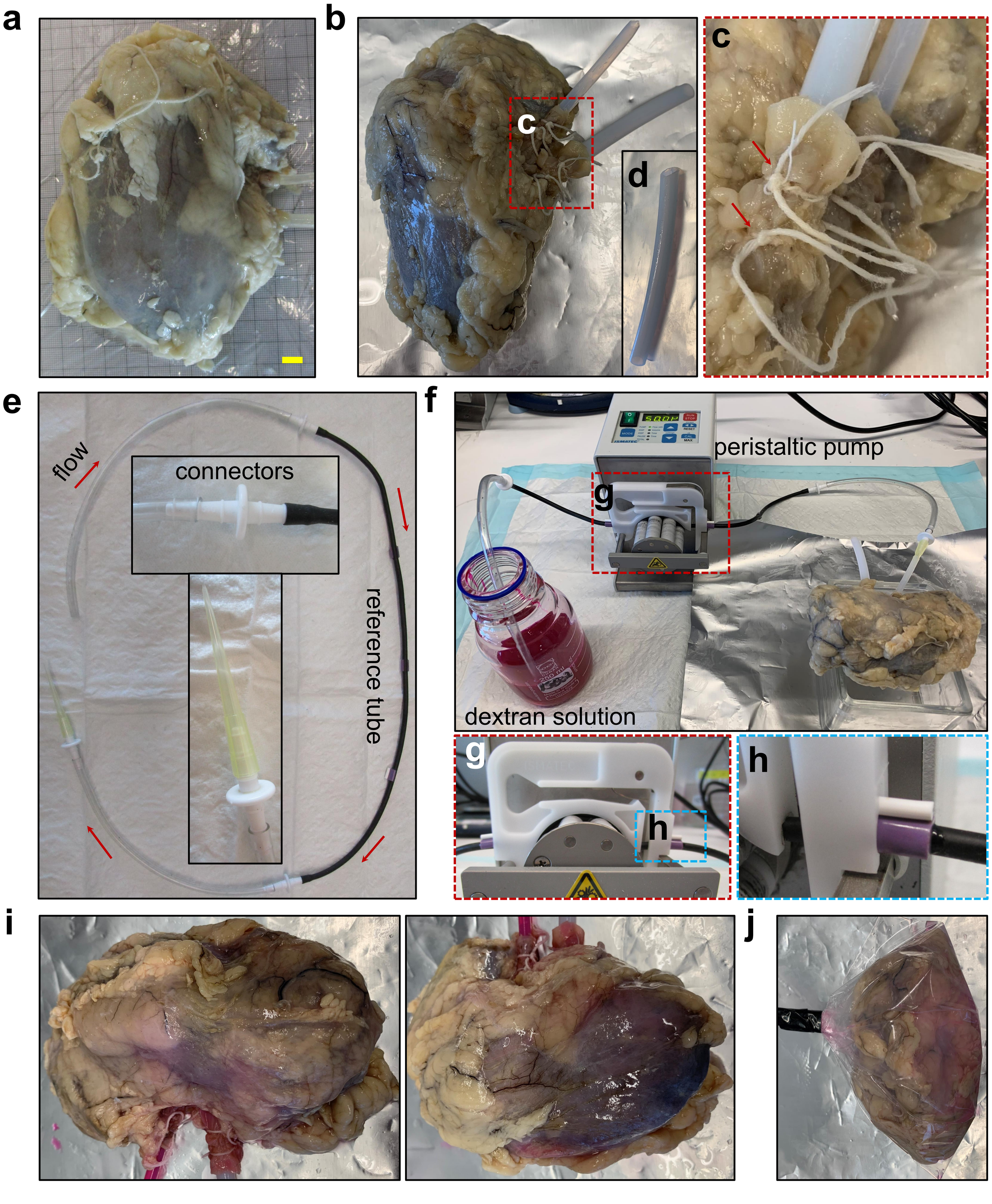


Figure 2. Vessel labelling with dextran solution by active perfusion
(a) Photo of dissected human kidney with inserted tubing (scale bar: 1 cm). **(b)** The tubing was changed to be PTFE (chemical-resistant, anti-adhesive, biocompatible) tube and tightly fixed by double rope-fastening (red rectangle in **c**). **(d)** The inserted nozzle of PTFE tube was cut with a slope angle to plug-in smoothly. **(e)** A set of connecting tubing contained the black pump reference tube in the middle, two white tubes at both ends, tube connectors and a pipette. The red arrows indicated the flow circulation direction. **(f)** Vessel labelling with dextran solution using pump perfusion system (rotation of the wheel in **g** enables solution flow into kidney, black pump reference tube was fastened by correct set up of cassette shown in **h**). **(i)** Photos of human kidney after dextran labelling. **(j)** The dextran labeled human kidney was sealed in a plastic bag.

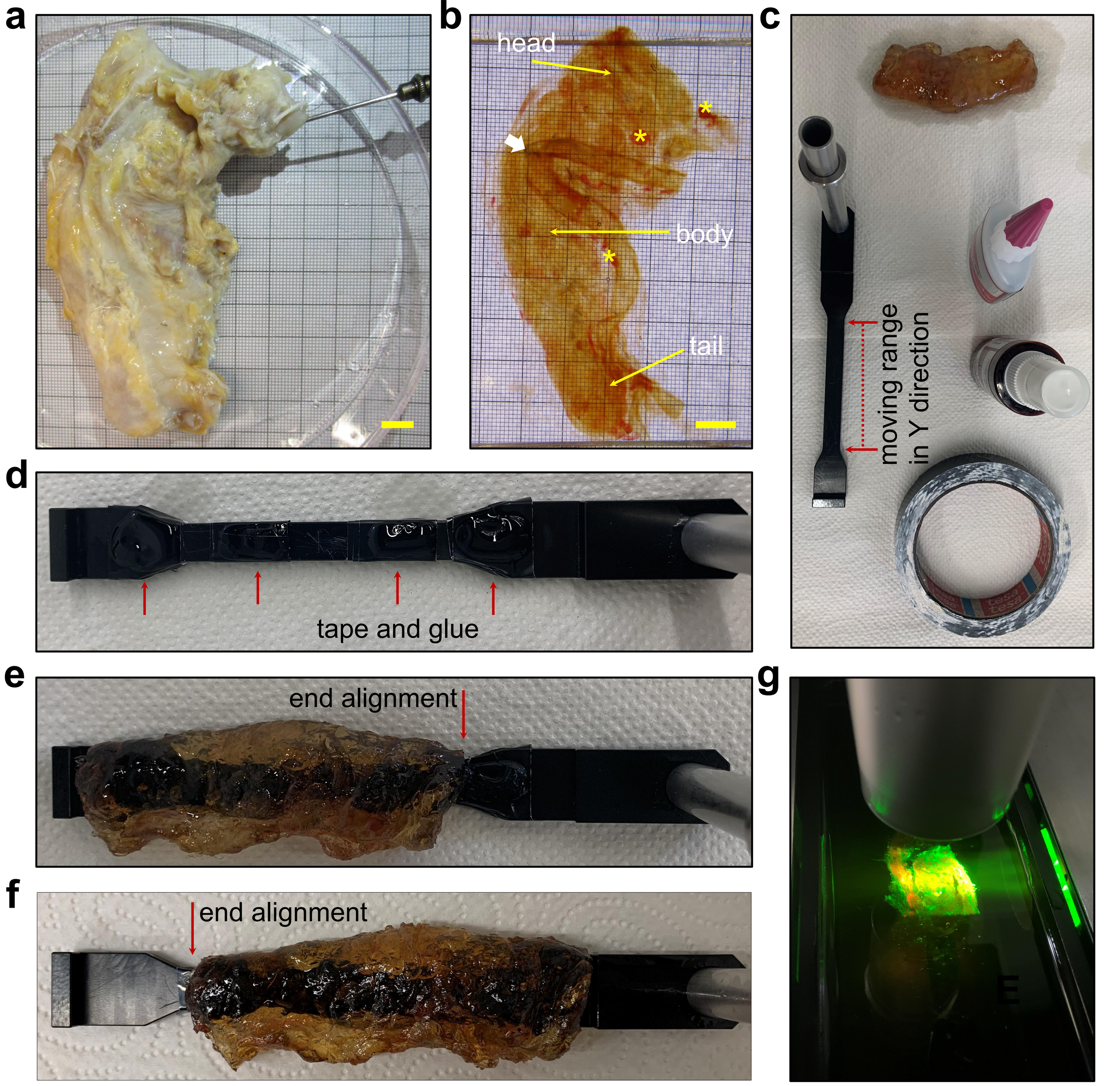


Figure 3. Organ mounting and imaging with light-sheet microscopy
(a) Photo of human pancreas before clearing (scale bar: 1 cm). (b) Photo of transparent human pancreas after PI cell nuclei labelling and clearing, showing mesenteric artery (white arrow) and lymph nodes (yellow asterisk) (scale bar: 1 cm). (c) The mounting of an organ on a sample holder required glue and black tape. The red arrows show the moving range ends of holder in Y direction. (d) The sample holder was gummed with four sections of tape and dropped with glue. (e) One example of sample position with one end alignment to cover one sample edge. (f) Another example of sample position with another end alignment to cover the other sample edge. (g) The mounted human pancreas was illuminated by light sheet.

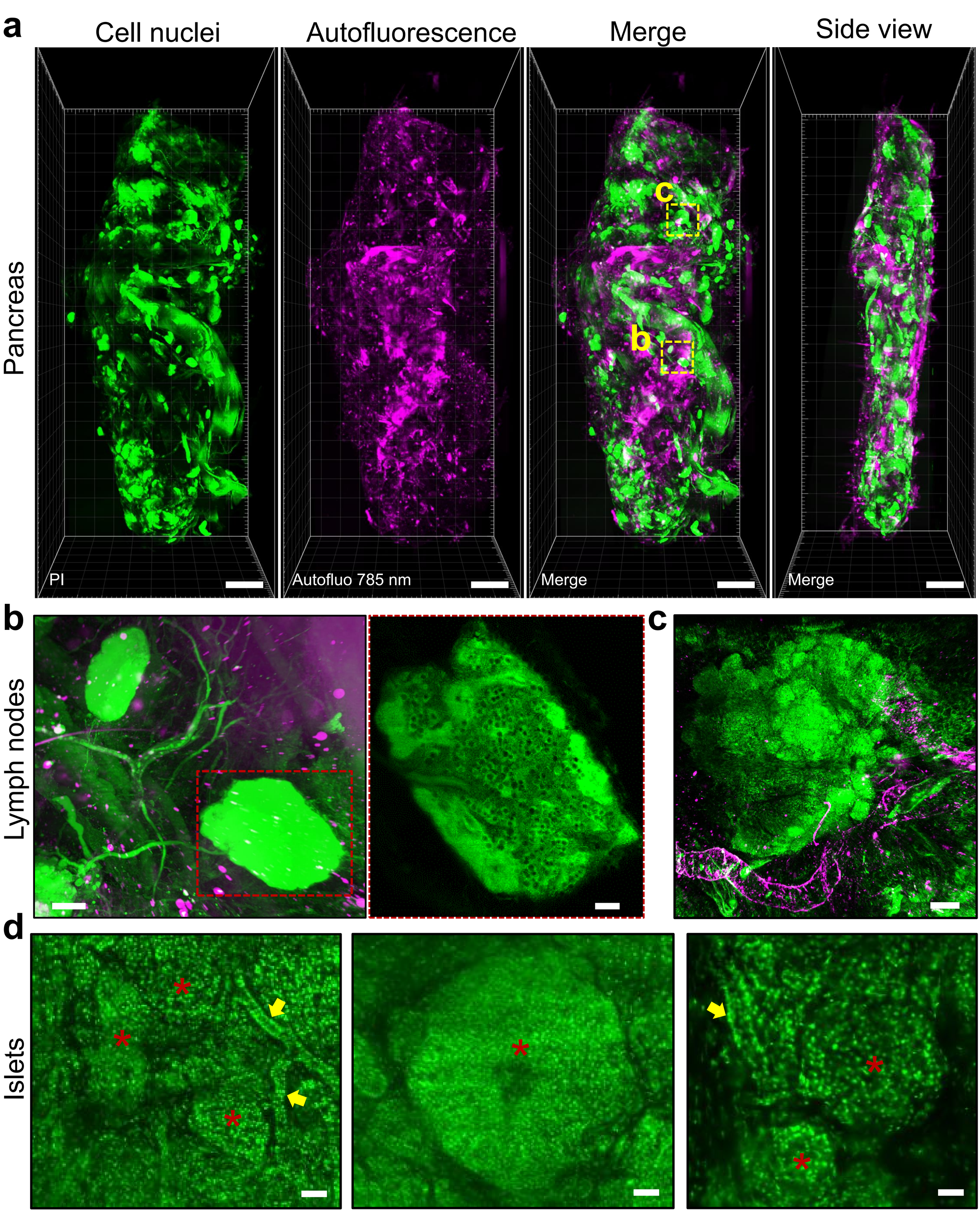


Figure 4. 3D reconstruction of human pancreas

(a) 3D reconstruction of human pancreas imaged by light sheet fluorescence microscopy focusing on the PI labeled cell nuclei (Green) and autofluorescence of 785 nm wavelength (magenta) (scale bar: 1 cm). (b) Zoomed 3D reconstruction in figure A showed the vessels and lymph nodes (red rectangular) (scale bars: 1 mm and 200um respectively). (c) 3D reconstruction of one example of endocrine portion by high magnification of 12X objective (scale bar: 200 um). (d) Diverse islets surrounded by acinar cells could be easily identified in 3D images of endocrine portion showing standard round or oval shape and irregular shapes as well as variable sizes (red asterisk). Also, intralobular ducts could be located near the islets (yellow arrow). (scale bars: 50 um, 50 um and 30um, respectively).

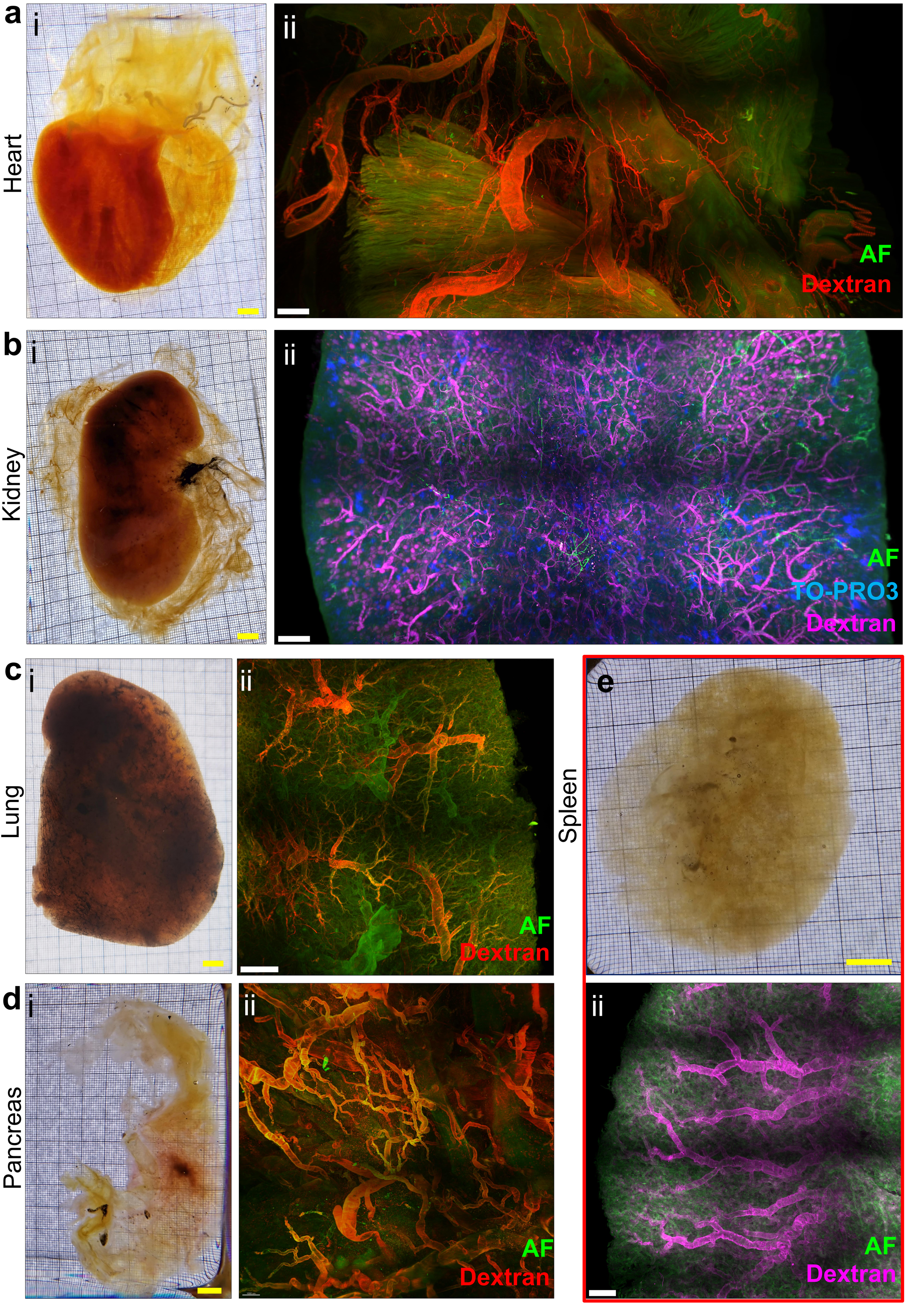


Figure 5. SHANEL of human organs with perfusion of dextran vessel labelling dye
(**a**) Human heart after SHANEL protocol (i), 3D reconstruction (ii) shows blood vessels of coronary artery (red) and myocardium which can be imaged in autofluorescence at 488 nm (AF, green) (scale bars: 1 cm and 2mm, respectively). (**b**) Human kidney after SHANEL (i), structure of glomeruli can be seen in dextran labelled. TO-PRO3 labelled cell nucleus (scale bars: 1 cm and 2mm, respectively). (**c**) One lobe of human lung was cleared and stained with dextran, bronchus can be seen in AF (scale bars: 1 cm and 2mm, respectively). (**d**) Image of human pancreas and vasculature after SHANEL (scale bars: 1 cm and 2mm, respectively). (**e**) Human spleen after SHANEL, fine structure of splenic artery was visualized in Imaris (scale bars: 1 cm and 2mm, respectively).

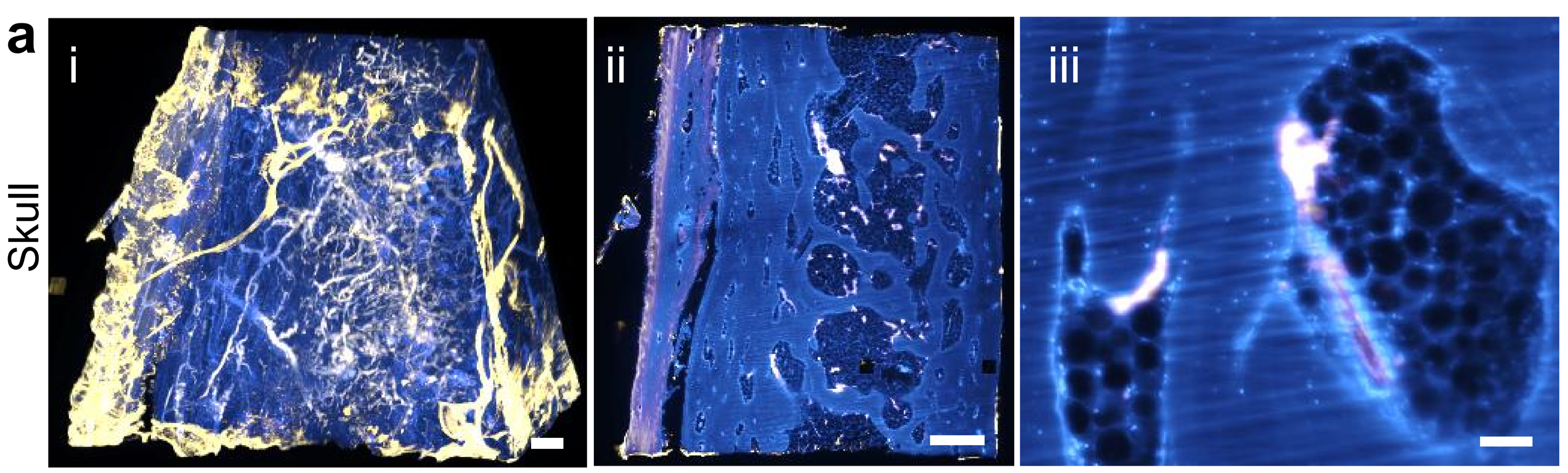


Figure 6. SHANEL of human tissues by passive incubation
(a) 3D reconstruction of skull labelled with lectin (yellow) and PI (blue) (i). XY section (ii) and magnified image (iii) (scale bars: 1mm, 1mm and 100um, respectively). (**b**) brain slice labelled with Neurotrace Nissl stain (scale bars: 2mm and 300um, respectively). (**c**) brain tissue labelled with Iba1 antibody (i). YZ (ii) and XZ (iii) section, magnified image in XY section (iv) (scale bars: 1000 um, 700 um, 500 um and 40 um, respectively). (**d**) human lung tissue labelled with α-SMA antibody (i). YZ (ii) and XZ (iii) section, magnified image in XY section (iv) (scale bars: 700 um, 500 um, 500 um and 150 um, respectively).

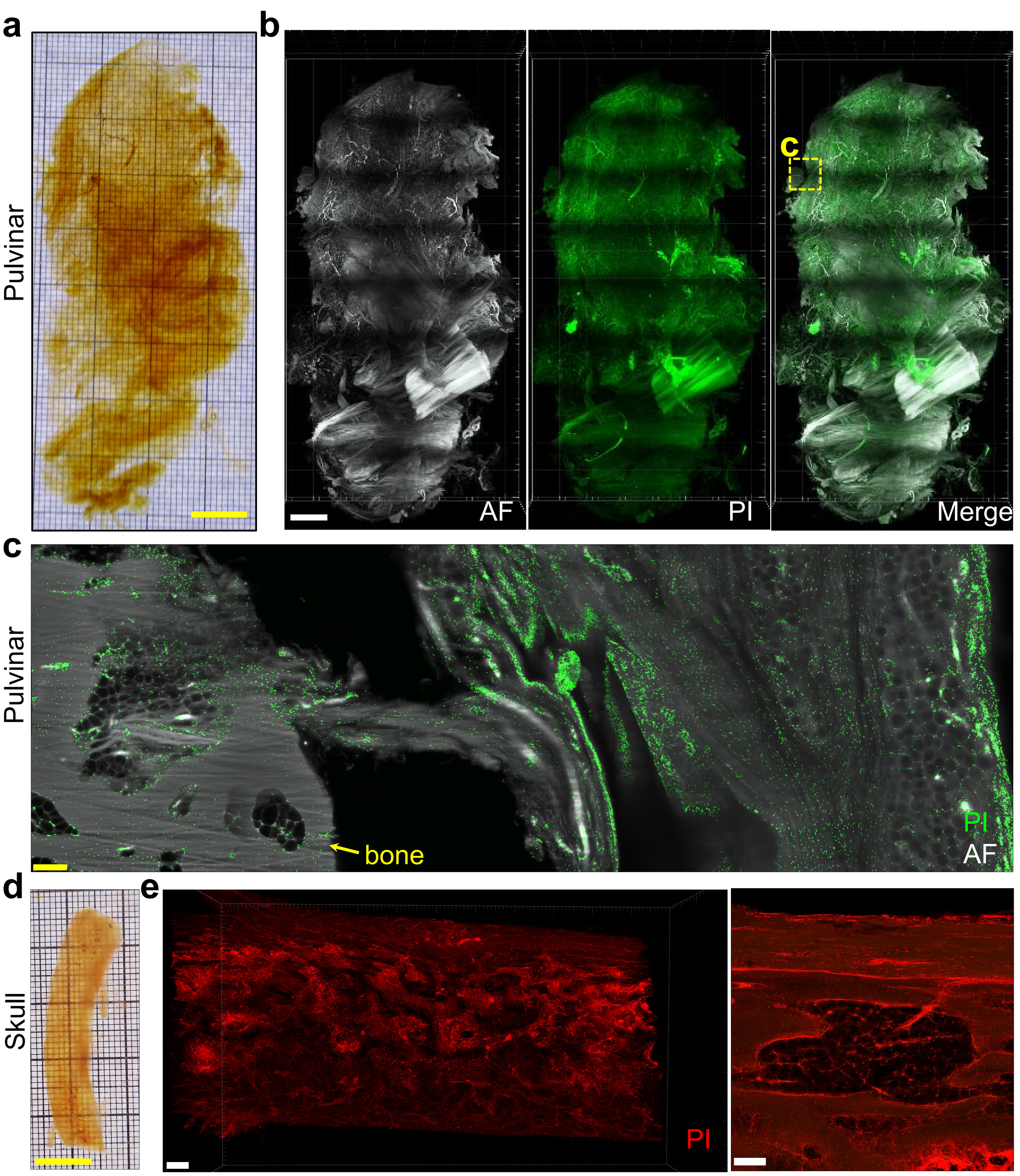
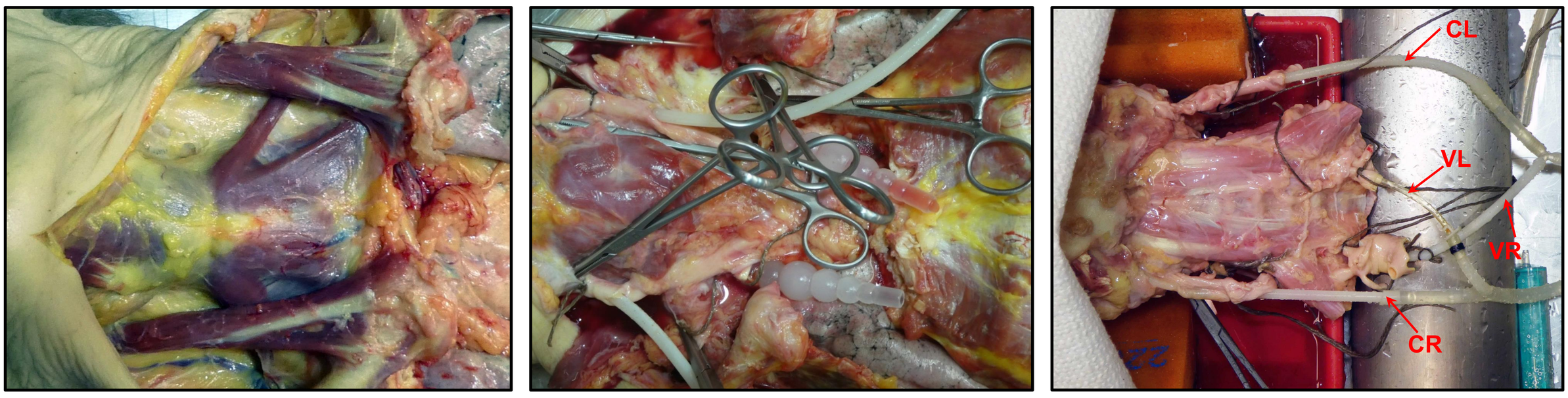
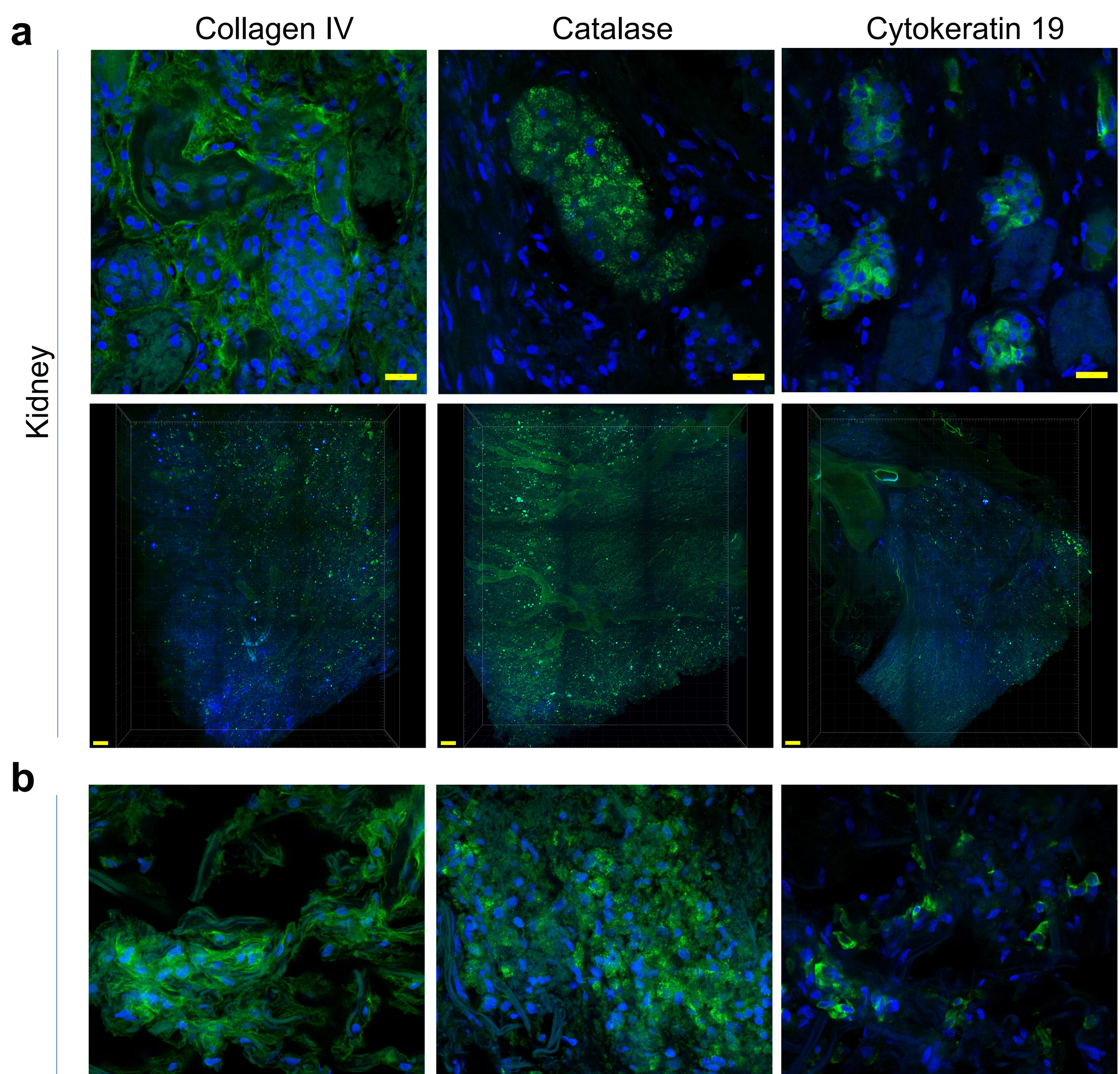


Figure 7. Passive incubation, clearing and 3D reconstruction of human pulvinar and skull
(a) Photo of transparent human pulvinar after PI cell nuclei labelling and clearing by passive incubation (scale bar: 1 cm). **(b)** 3D reconstruction of human pulvinar imaged by light-sheet fluorescence microscopy focusing on autofluorescence (AF) of 488 nm wavelength (grey) and PI labeled cell nuclei (Green) (scale bar: 1 cm). **(c)** section view of pulvinar showing the connection between bone tissue and the pulvinar fibrofatty tissue (scale bar: 150 um). **(d)** Human skull bone after PI cell nuclei labelling and clearing (scale bar: 1 cm). **(e)** The human skull imaged by light-sheet and confocal microscopy (Scale bar 500 um and 200 um, respectively).

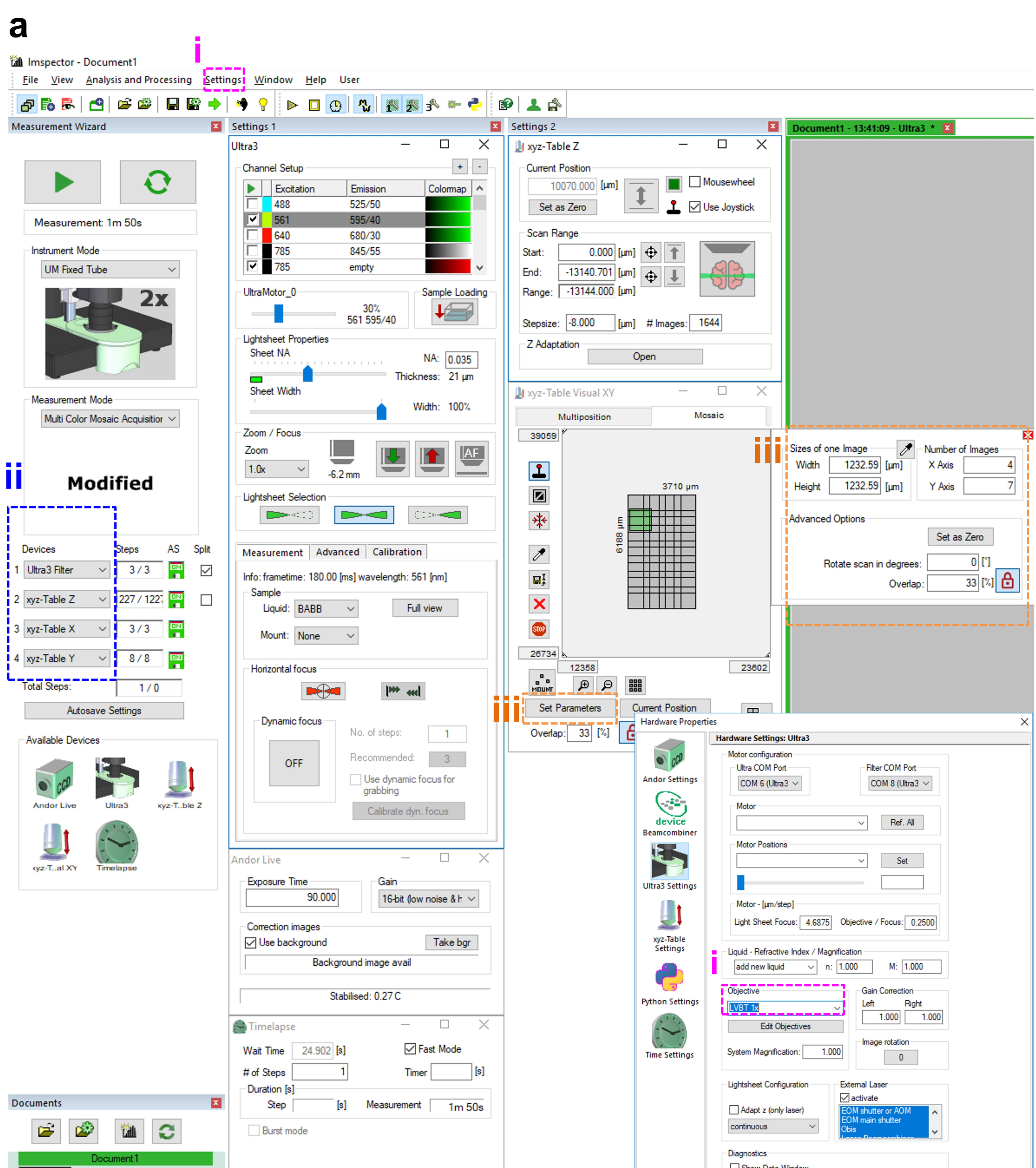


Supplementary Figure 1. Human brain clearing by perfusion system
(a) The dissection of human brain with CL and CR (left and right carotids, respectively), VL and VR (left and right veterbral arteries, respectively). **(b)** The human brain after dissection and fixation. **(c)** The set up of human brain clearing by perfusion system under fume hood. The human brain was put in a glass container and connected with input and output tubing controlled by peristaltic pump. There were four channels to control the four connecting tubings as indicated in C i. Two output tubings were connected with the carotids arteries as shown in C ii and two output tubings were connected with the vertebral arteries as shown in C iii. The input tubings were protected with gauze to avoid solid impurities entering and blocking the tubings (see C iv). C v showed the decolorization effects of CHAPS/NMDEA according to the blooming of dark green color from the organ. **(d)** The glass container was sealed with several layers of plastic wrap to prevent the volatility of running solutions, especially for EtOH, DCM, MeOH. **(e)** Photo of human brain after SHANEL pretreatment before clearing. Remaining blood in the vessels (**b**) was decolorized.



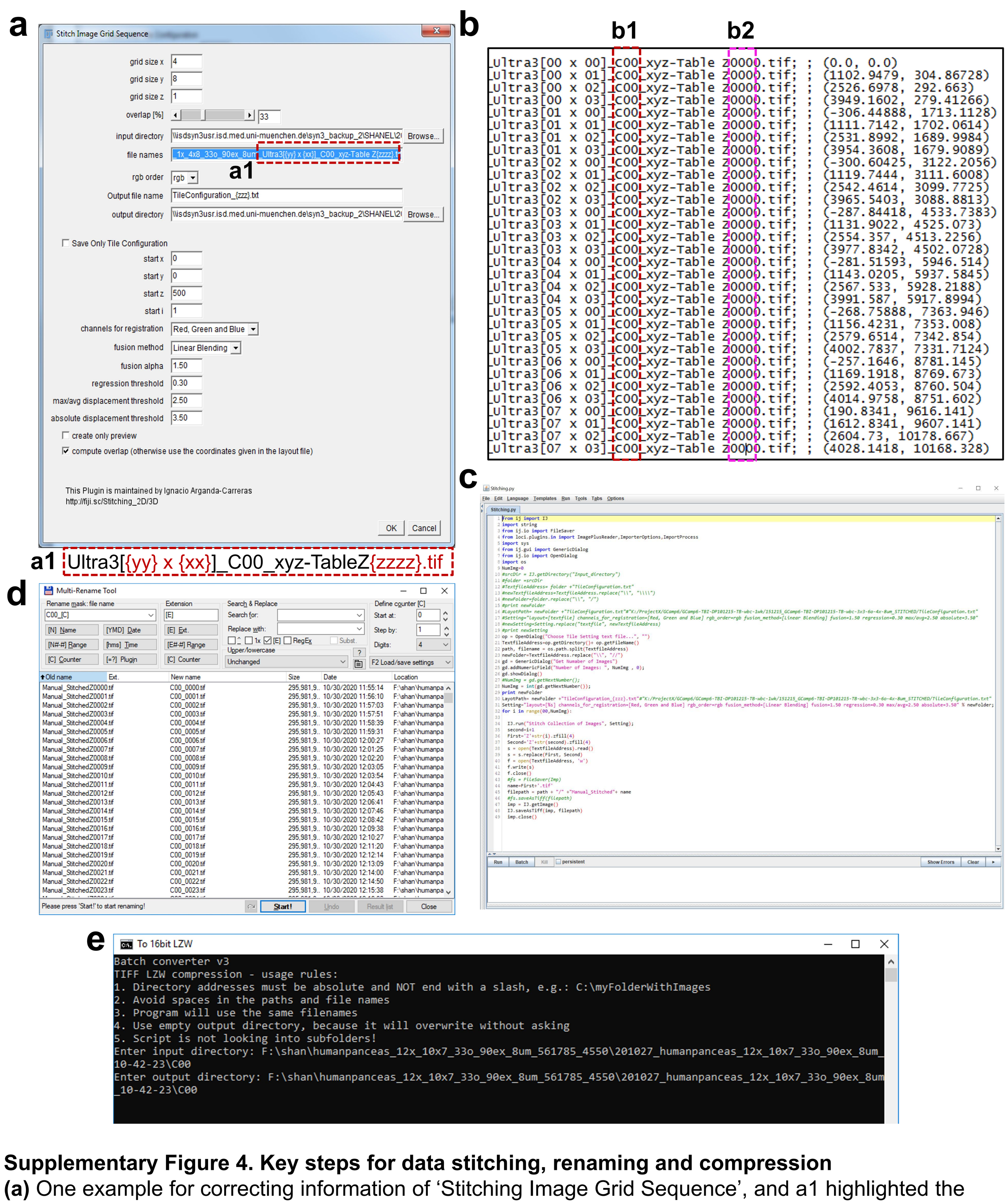
Supplementary Figure 2. Passively stained conjugated antibody in centimeter-size human kidney and lung tissue

Collagen IV, catalase and cytokeratin 19 antibody staining (Green) in human kidney (**a**) and lung (**b**). Top panel shows immunofluorescent staining images in confocal microscopy to verify antibody compatibility with SHANEL. Bottom panel shows 3D reconstruction of tissue with antibody labelling in Imaris. (scale bars: 20 um and 500 um, respectively)

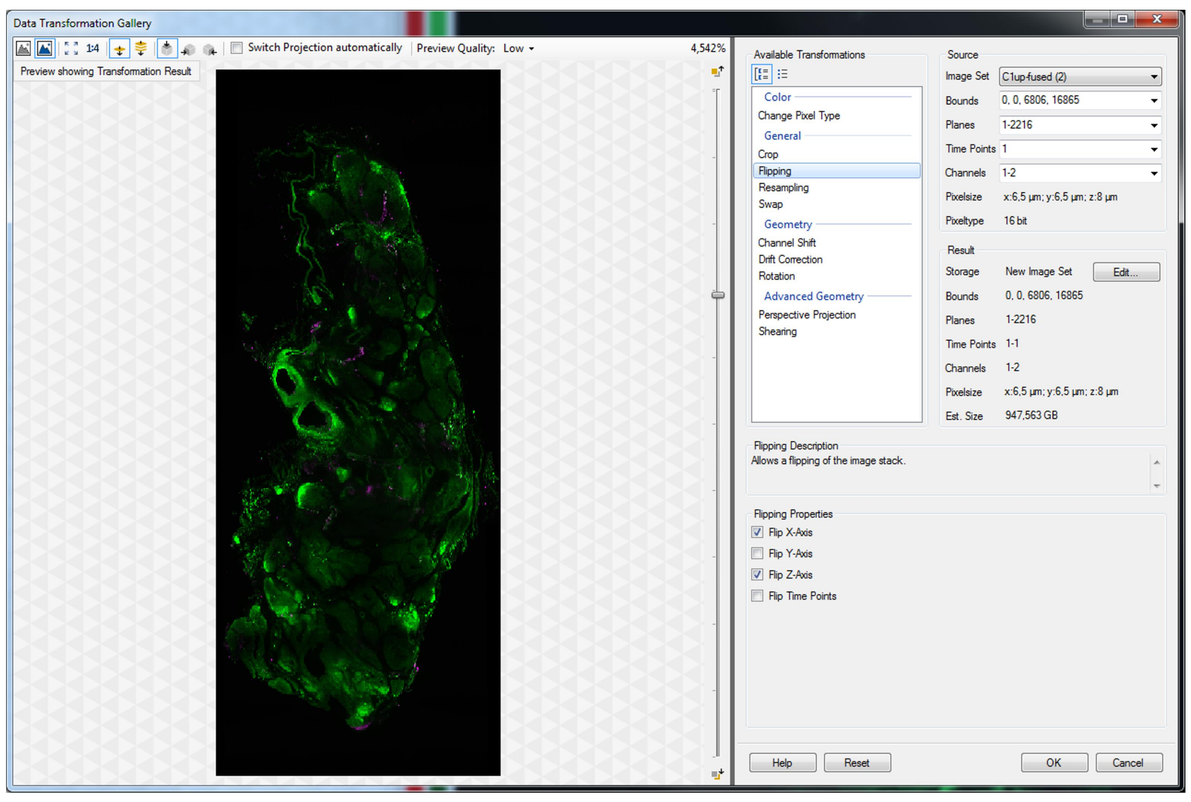


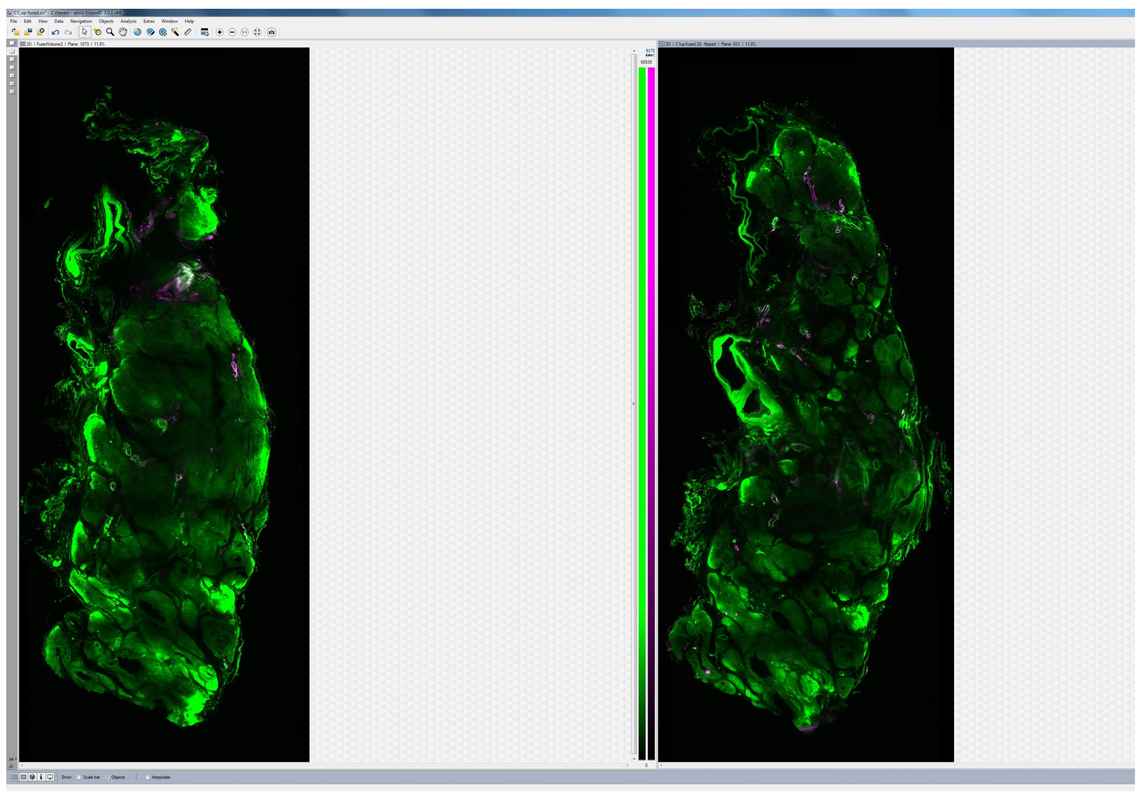
Supplementary Figure 3. Settings in imaging software

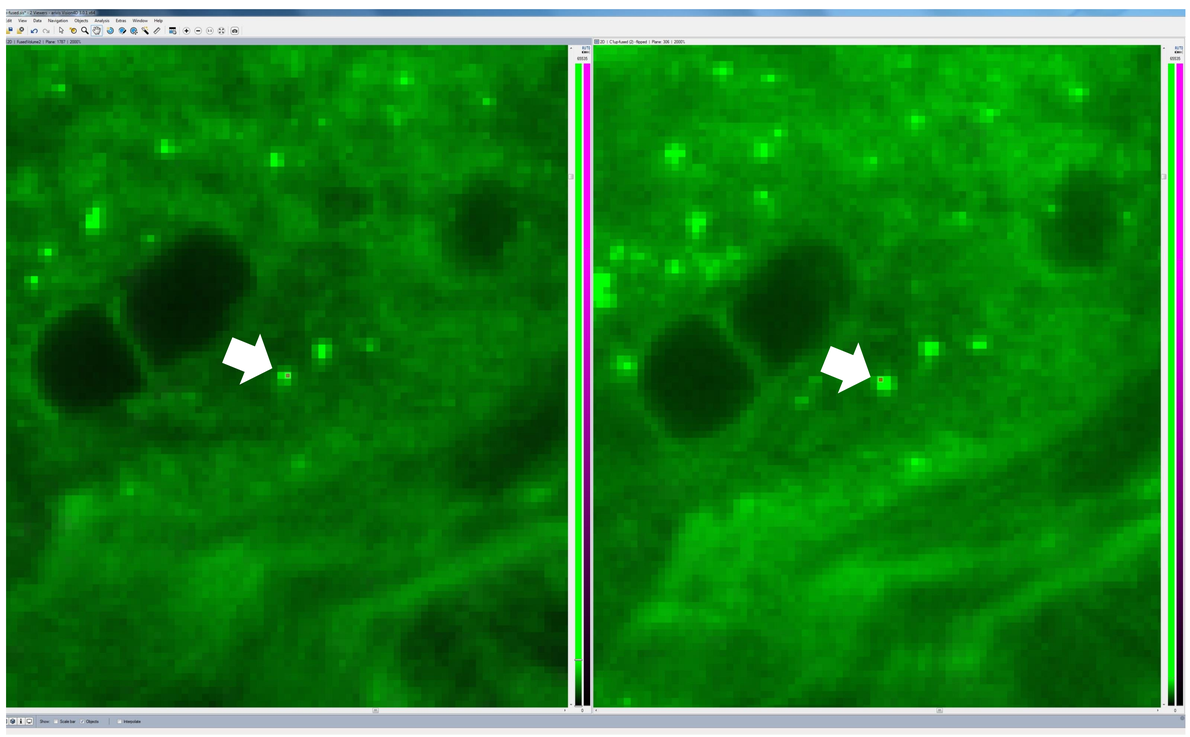
(a) The user interface of Lavision Imspector software. (i) refer to the setting of objective lens and magnification. **(ii)** The names of the devices should be listed in the correct order. **(iii)** One example for settings of the tiling scan with 4x7 tiles and 33% overlap.

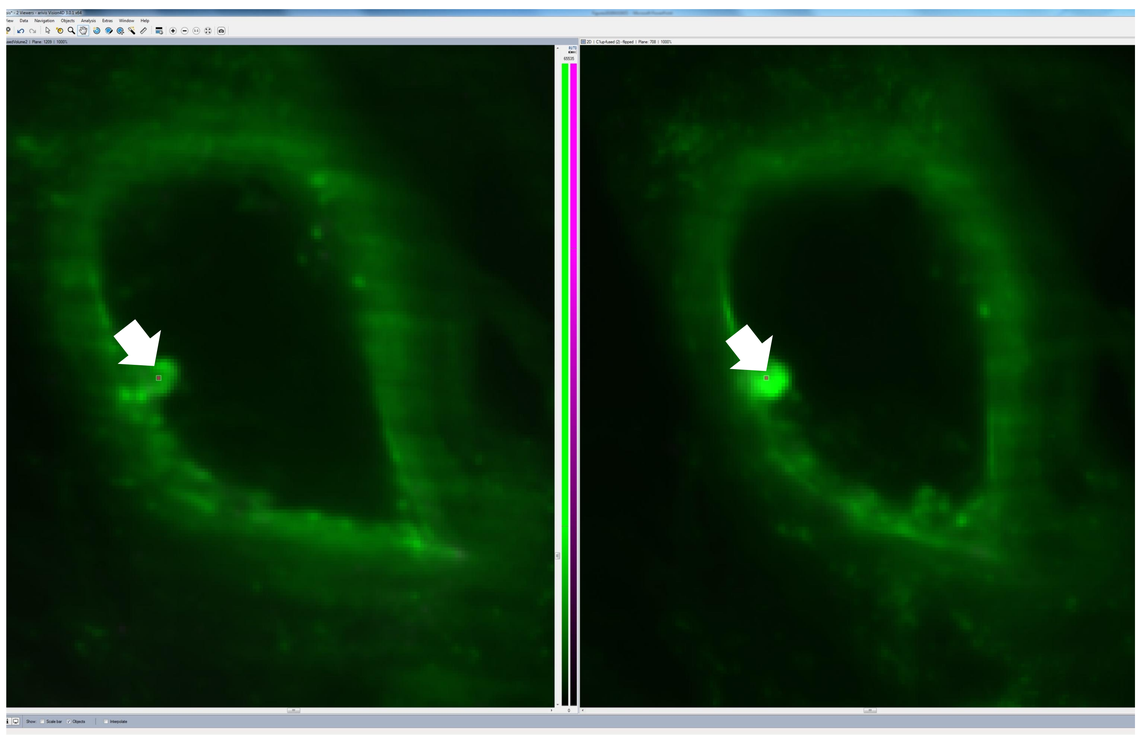


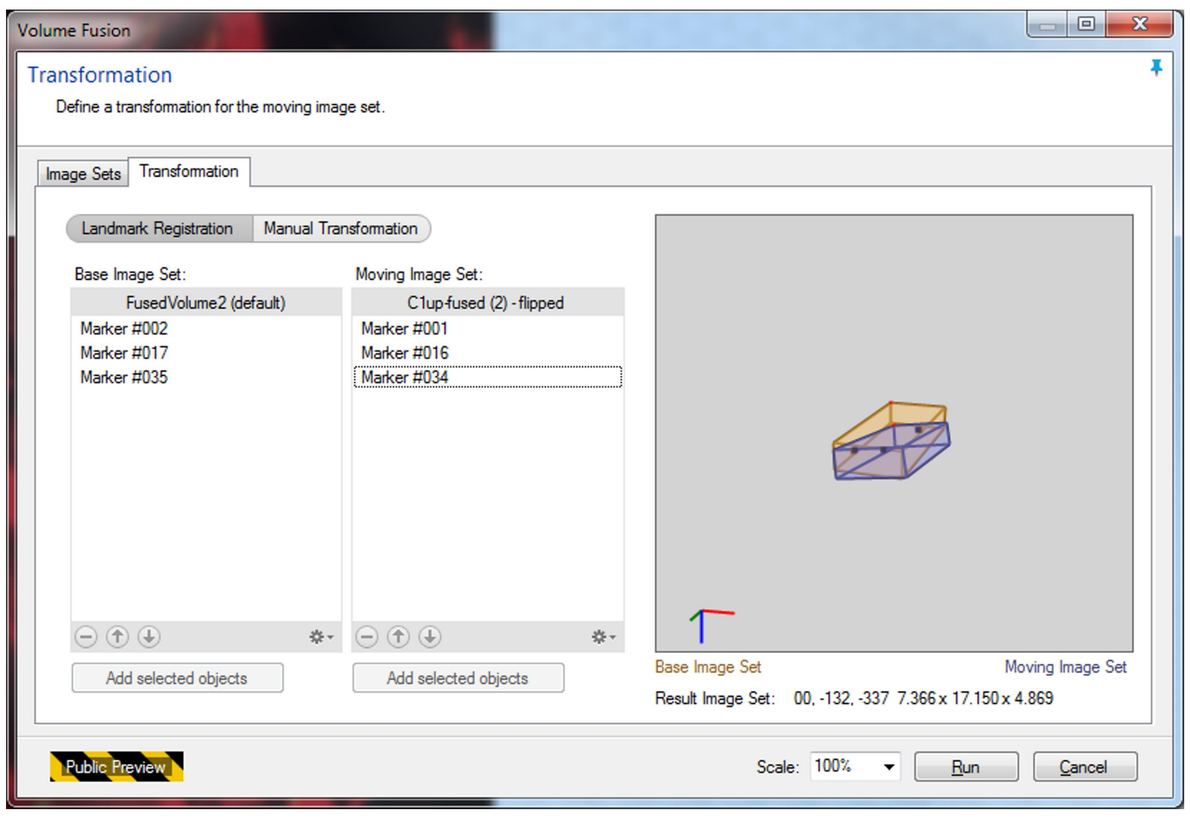
Supplementary Figure 4. Key steps for data stitching, renaming and compression
(a) One example for correcting information of ‘Stitching Image Grid Sequence’, and a1 highlighted the replacement of red letters in step x about ‘file names’. **(b)** The key information of ‘TileConfiguration’ txt file, b1 highlighted the responding channel number should be corrected and saved in respective separated folder and b2 highlighted the starting Z number should be ‘0000’ for all ‘TileConfiguration’ files. **(c)** The information of ‘Stitching.py’. **(d)** One example for settings of ‘Multi-Rename Tool’. **(e)** One example of compressing tiff files with ‘LZW TIFF’.

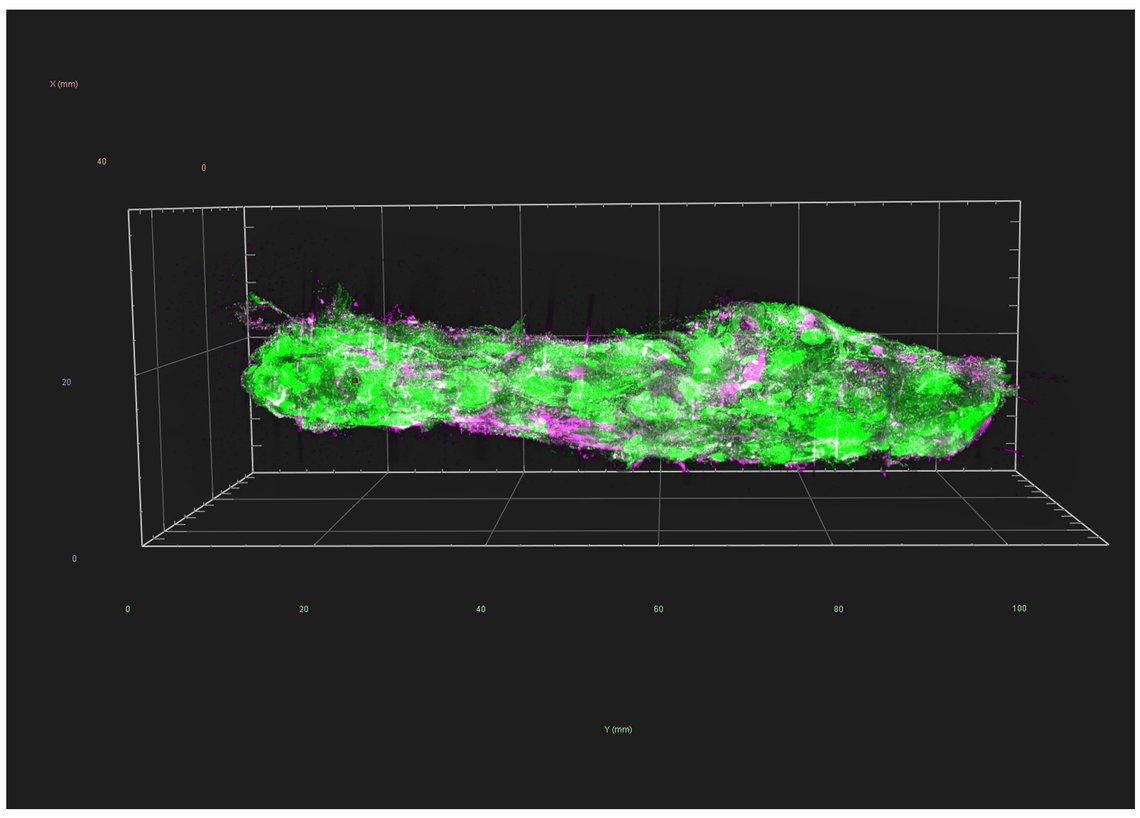






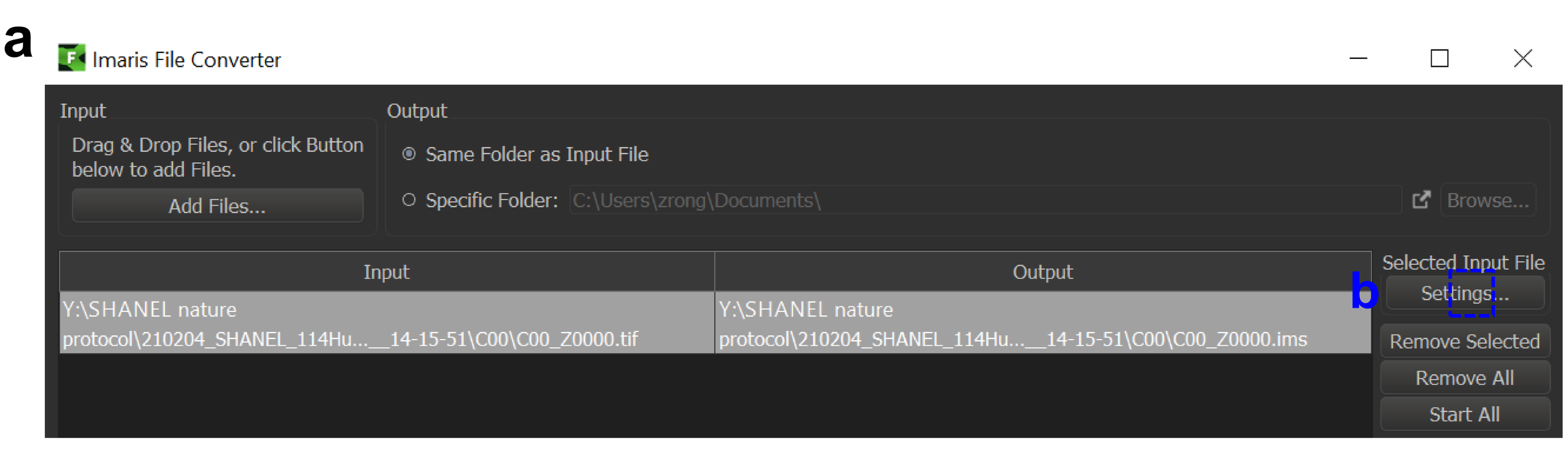


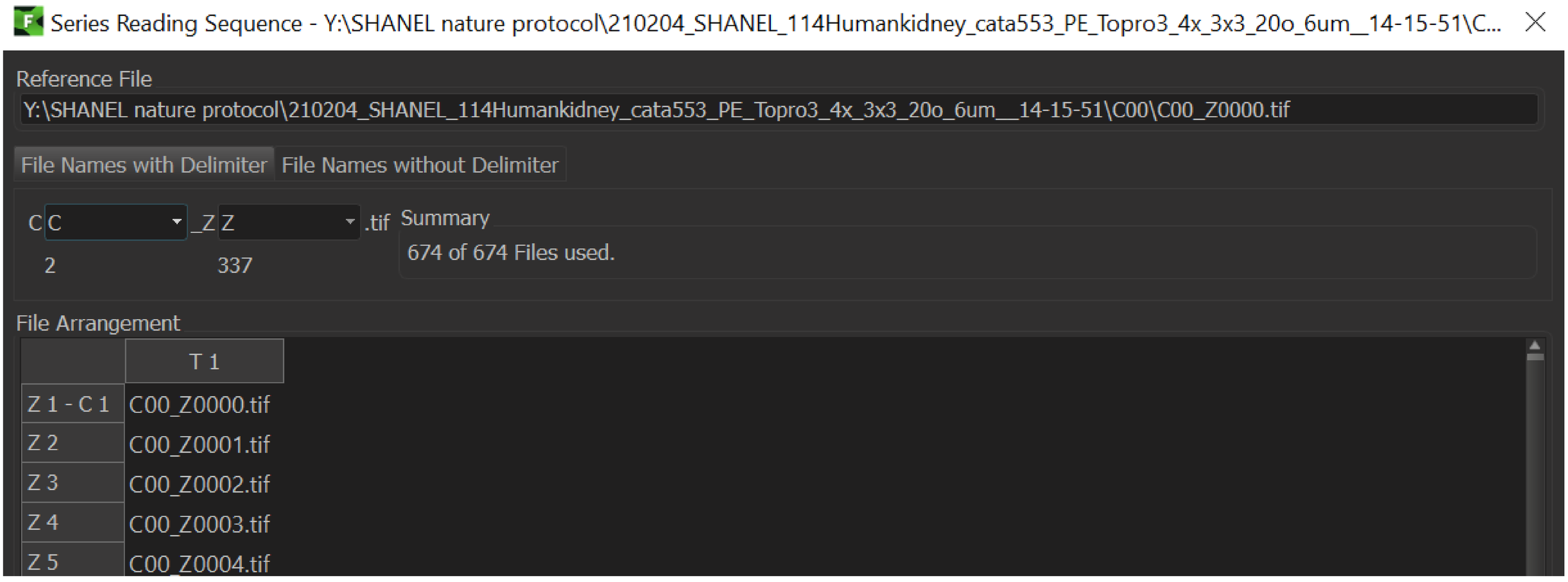


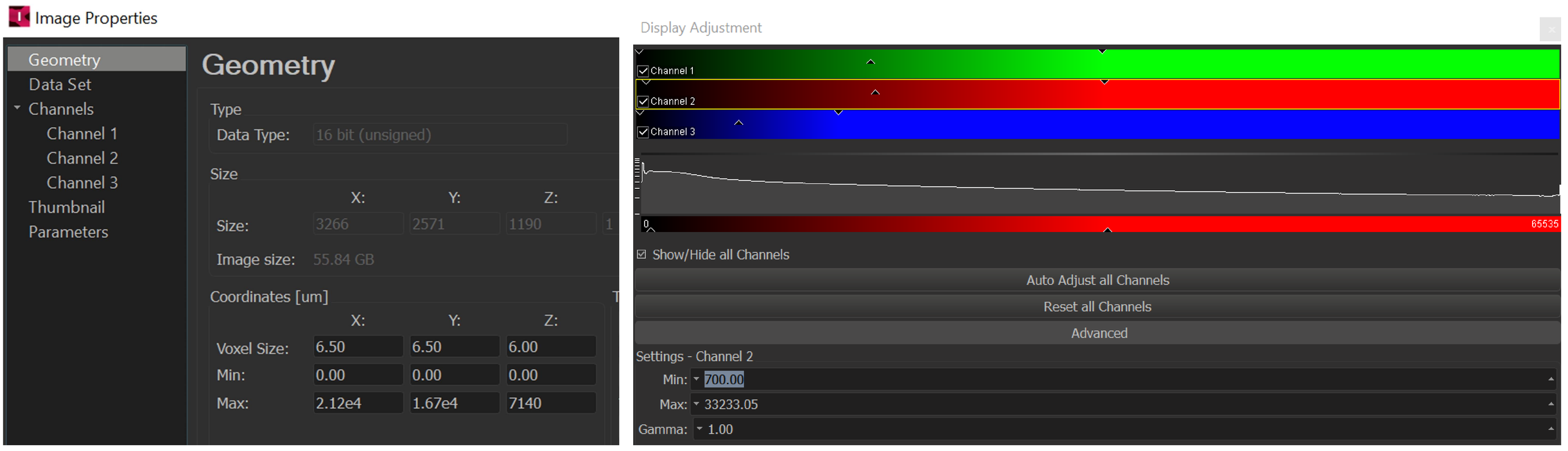


Supplementary Figure 5. Key steps for Arivis fusion of 3D images

(a) One example of flipping one volumetric data of X or Y or Z direction to match with another volumetric data. **(b)** The overview of two volumetric data showing same XYZ directions after flipping. **(c-d)** Examples of identical structural markers from two volumetric data sets for fusion. **(e)** Loading of three key markers for best fusion. **(f)** 3D fused image of pancreas from two volumetric data sets.

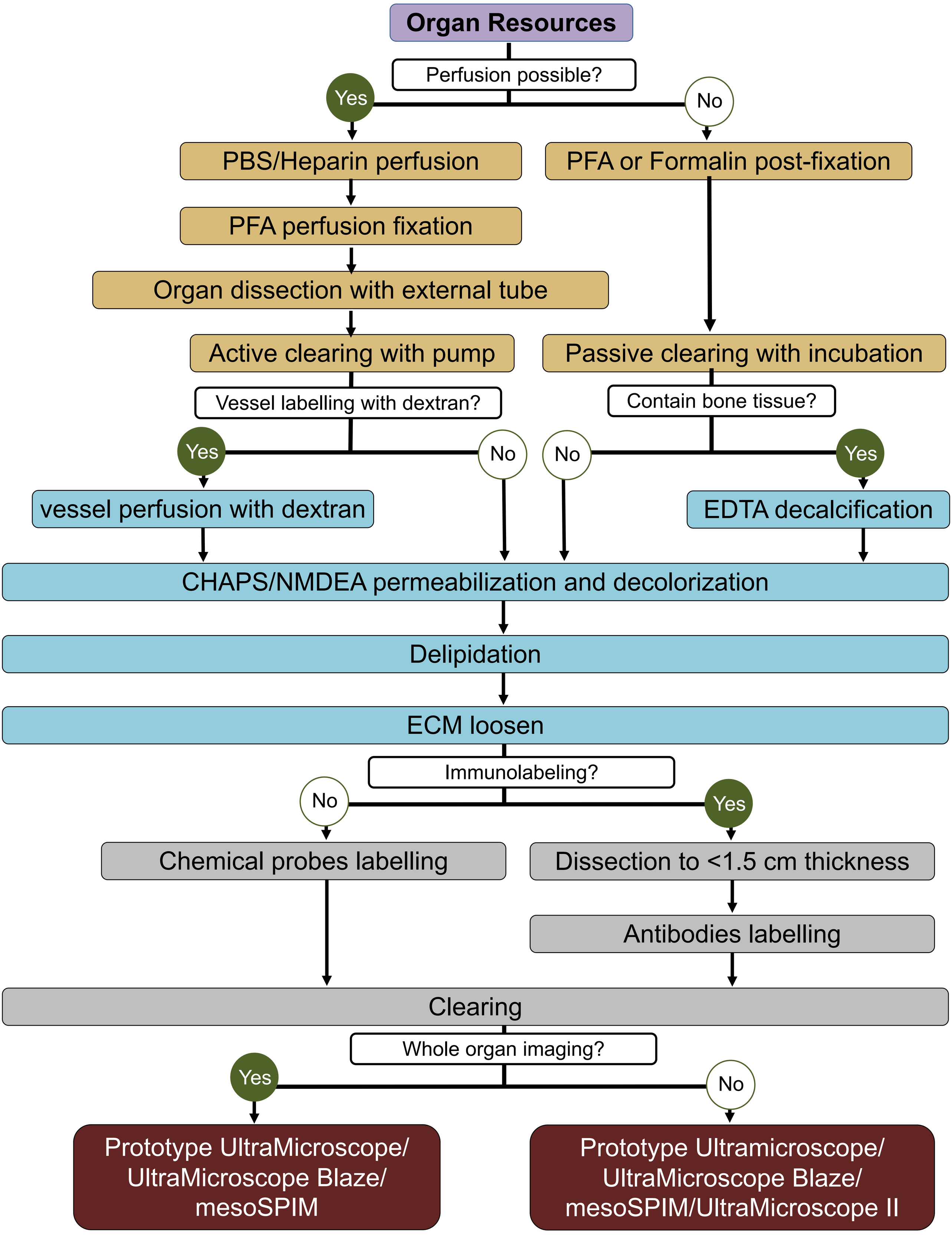






Supplementary Figure 6. Key steps for Imaris data loading and visualization

(a) One example of converting stitched image sequence data to the .ims format using Imaris File Converter. (**b**) The settings for file names with delimiter should be changed into “C _ Z.tif”. (**c**) 3D image properties including XYZ voxel size and channel color can be set in Imaris for analysis.



Supplementary Figure 7. Overview of SHANEL workflow
This workflow summarizes the main steps after organ collection: experimental setup (yellow), pretreatments (blue), labelling and clearing (grey) and imaging (red). We provided two ways of handling samples: active perfusion with pump, if the vessels could be connected with external tube, and passive incubation with shaker. Moreover, depending on the organ components, organ size, targeted labelling, and imaging structurers, we outlined the differences in the whole process.