Single-cell level imaging of whole mouse body with vDISCO

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Abstract

Homeostatic and pathological phenomena often show effects on multiple organs and whole organisms. Tissue clearing methods have made a holistic examination feasible together with recent advances in clearing and microscopy. Here, we report the detailed protocol for vDISCO, a highly reproducible pressure-driven, nanobody-based whole-body immunolabeling and clearing technology that renders whole rodents transparent in 3-4 weeks, enhances the signal of fluorescent proteins and stabilizes them for years. This allows the reliable detection and quantification of fluorescent signal in intact transparent rodents and permits an unbiased and *in toto* detailed analysis of an entire body at cellular resolution. Importantly, we show the high versatility of the method: how vDISCO can be applied to boost the signal and clear different dissected organs and tissues and how vDISCO processed samples can be imaged with multiple fluorescence microscopy systems. In addition to its applications in obtaining a whole-body mouse neuronal map, detecting single-cell metastases in whole mice and discovering new anatomical structures as we showed previously, here we report the characterization of the entire mouse lymphatic system, the application for virus tracing and the visualization of all pericytes in the brain. Taken together, our vDISCO pipeline represents a powerful tool to conduct systematic and comprehensive studies of the cellular phenomena and connectivity in intact bodies.

Introduction

As most biological tissue is opaque, the process of sectioning tissues and organs in histology for microscopy analysis has represented for a long time an inevitable burden. Sectioning is time consuming, error-prone, tedious and most of all it forces the scientist to select a particular tissue or organ of interest for the study, possibly overlooking critical events located in other body parts. This aspect is particularly important when many diseases affect different organs and often the whole body, therefore an unbiased way to analyze the entire organism is needed[1]. Evidently the sectioning of a small mammal such as a rodent into 5-100 µm thick slices for the analysis of the entire body would be almost unfeasible, while other whole body imaging techniques such as PET or MRI do not achieve sufficient resolution to detect morphological changes at subcellular level[2–4]. Particularly, in neurobiological studies the possibility to have a quick access to the 3D structures of complex brain cells such as microglia and neurons is extremely advantageous because their morphological reconstruction would not be based on reconnecting their fragments observed in each slice[5]. The development of tissue clearing[5–10] methods, deep-tissue immunolabeling techniques[11–14]and the maturation of light-sheet microscopy[15] in the last 15 years allowed the adoption of a more holistic histological approach to study the structures of tissues and organs: since the sample was made transparent, the 3D information was quickly and readily accessible. In the last years, tissue clearing was applied to make even the whole body of adult mice transparent after the removal of the skin[16–22], paving the way to the concept of whole-body 3D histology. However, these clearing protocols relied on the detection of transgenically expressed fluorescent proteins including green fluorescent protein (GFP), enhanced yellow fluorescent protein (EYFP) and mCherry. Although these proteins were commonly used in fluorescence imaging, when imaged in whole body they were often not sufficiently bright to overcome the strong autofluorescence from the surrounding tissues such as muscles, calcified bones and the pigment-rich skin. In addition, they were eventually quenched by the clearing reagents[16] and bleached quickly during an imaging session, thus rendering sample re-imaging often impossible. All these factors led

to a problematic and not reliable detection of the signal and consequently a difficult and sometimes impossible quantification of the fluorescent structures. Therefore, we sought to develop a new pipeline called vDISCO (nanobody(V_HH)-boosted 3D imaging of solvent-cleared organs)[23] (**Figure 1**) that can overcome the problems above-mentioned and that can finally allow easy and reliable head-to-toe imaging of an entire organism at subcellular resolution.

Development of vDISCO

We were interested in a method that could achieve a reliable detection of fluorescent structures in thick samples such as whole rodent bodies (**Figure 2**) for subsequent quantitative analysis. Transgenically expressed fluorescent proteins are widely employed in biomedical research; however, their signal in histological applications in combination with widefield microscopy is often too dim even when cut in a few hundred micrometer thick sections. This is due to the fact that in histology the fluorescent proteins are embedded in the tissue, a complex mesh of biological components that constitute the background signal. This background causes light-scattering[24,25] and emits autofluorescence[26,27]. Understandably, the amount of tissue in a whole body increases the background signal, with the result of diminishing the signal-to-background ratio. This represents a further considerable obstacle for the detection of the fluorescent proteins. The process of tissue clearing, since it homogenizes the refractive index throughout the tissue, helps reduce the light-scattering and therefore decrease the opacity of the tissue[10,24,25,28]. Nonetheless, tissue clearing alone does not necessarily decrease the light absorption and the intrinsinc autofluorescence emitted from endogenous pigments in the tissue[29–31].

In standard histology the use of antibodies targeting the fluorescent proteins and conjugated to bright synthetic fluorophores (such as the ones from Alexa and Atto family) represents a solution to increase the brightness and visibility of the proteins. Therefore, we reasoned to use this strategy to increment the signal of fluorescent proteins via whole-body immunolabeling. Our aim was to not only enhance the signal contrast (signal-to-background ratio) and stabilize the fluorescent signal, but also to shift the emission spectrum to far-red, a channel tha[32,33]t is characterized by less biological autofluorescence and that allows better penetration of the excitation light due to its longer wavelength.

Since antibodies are relatively big proteins (~150 kDa) and thus cannot penetrate in histological preparations for more than a few hundred micrometers (unless with the use of harsh chemical pretreatments such as with methanol[11]), we decided to employ nanobodies to achieve whole-body immunolabeling. In fact, nanobodies represent the smallest entities derived from immunoglobulins that are able to bind an antigen, being 10 times smaller than antibodies (12–15 kDa)[32,33] . Moreover, they are conjugatable to synthetic fluorophores[34]. In addition, we further increased the penetration efficiency of the nanobody into the tissue by increasing the incubation temperature of the sample with the nanobody to 37°C[11,35], by using *N*-acetyl-L-hydroxyproline to destabilize the tissue collagen[10] and by adding methyl-β-cyclodextrin to extract the cholesterol[10]. The last two chemicals could render the extracellular matrix more permeable.

Previously, we verified in dissected brains from CX3CR1-GFP[36] mice (expressing GFP in microglia) that the passive staining with nanobodies was indeed more efficient in boosting the fluorescent GFP signal compared to antibodies in whole brains[23]. Besides, we also found out that synthetic fluorophores were extremely stable after clearing, being very bright up to some years after labeling[23] (**Figure 2,3**). However, we promptly realized that the only passive incubation of the whole mice with nanobodies was still not

sufficient to achieve an extensive labeling of the fluorescent proteins in a volume that was greatly larger than single brains[23]. We then decided to exploit the cardiovascular network of the fixed animals to homogenously push the nanobodies in all body districts by using a high pressure perfusion system (160–230 mmHg compared with 70–110 mmHg of standard perfusion protocols[37]) (**Supplementary Figure 1**, **Figure 4,5**). Furthermore, already before the immunolabeling step, we additionally decreased sources of autofluorescence and light-scattering by adding a decolorization step, where the animal was perfused with chemicals that eliminated the remaining heme pigment from the blood, and a decalcification step, where the animal was perfused with a solution containing Ethylenediaminetetraacetic acid (EDTA) to extract the calcium from the bones. A ¼ dilution of CUBIC reagent 1[18] was chosen as decolorization chemical, because extensive chemical screening showed the efficiency of the aminoalcohols[38] such as N,N,N′,N′-tetrakis (2-hydroxypropyl)ethylenediamine (Quadrol) in CUBIC reagent 1 (CUBIC#1) to elute the heme from the tissues[18]. At the same time, EDTA was chosen as decalcification reagent, because of its well-known ability of sequestering Ca^{2+} ions and its compatibility with many other tissue clearing methods such as Bone Clarity[39] or PACT-decal[21]. In the end, the clearing process of both single organs and whole bodies was performed with a passive version of 3DISCO clearing[5,40] which used Tetrahydrofuran (THF) to dehydrate the sample, dichloromethane (DCM) to delipidate the sample and a mixture of benzyl alcohol + benzyl benzoate 1:2 (BABB) to render the sample transparent. 3DISCO was chosen for its high reproducibility, speed (clearing of a whole mouse in 4-5 days), good transparency and ease because it simply required sequential exchanges of different solutions. Owing to the combination of all these factors, vDISCO could render the fluorescent signal from transparent bodies highly visible through bones (e.g skull) and muscles, allowing reliable detection and quantification of fluorescent structures including cancer cells[1] **(Figure 2b-g),** microglia and neurons[23] (**Figure 3**).

Advantages and applications of vDISCO and comparison with other methods

In the last years, whole-body mouse clearing and imaging have been achieved by different protocols[16–22]. However, since these methods are based on the detection of transgenically expressed fluorescent proteins, they provide low signal contrast which can hinder reliable quantifications of fluorescent signal on such big samples. For example, CUBIC-cancer[19] and PEGASOS[22] protocols could achieve clearing of intact mice, although the quantification of the data shown in the papers was done on dissected individual organs on images obtained with confocal or 2-photon microscopy. Intuitively, the possibility to perform quantification directly on whole transparent bodies would represent a significant step forward to fully take advantage of whole-body clearing and imaging.

Instead, our vDISCO pipeline represents the first method that achieved whole-body immunolabeling, clearing and subcellular resolution light-sheet imaging of entire rodent bodies, leading to the reliable quantification of biological fluorescent structures in intact organisms[1,23]. This was made possible by several characteristics of vDISCO that provided different advantages, with the final result of improving the signal over background ratio.

The decolorization step achieves this by removing the residual heme in the sample. This step especially improves the clearing in organs rich of blood, such as liver and spleen.

The decalcification step provides the same advantage by enabling the clearing of bones through reducing the light scattering. This results in organs enclosed into bones such as the brain into the skull become optically accessible without the need of dissecting them, preserving in this way all the tissues therein located. For instance, the confirmation of the presence of brain lymphatic vessels in the meningeal

compartment[41] has always been troublesome since the standard procedure to dissect the brain from the skull for histological examinations could damage the meninges. With vDISCO, we could directly image this area and visualize these vessels[23,42].Previously we showed that the boosting step by using nanobodies conjugated to synthetic and bright fluorophores could enhance the signal over background ratio up to 2 orders of magnitude[23] and could shift the fluorescent signal to far-red spectrum when dyes as Alexa647 or Atto647N were chosen. The far-red range is quite beneficial because it increases the light penetration into the tissue and it is characterized by a lower autofluorescence[26]. Thus, small cellular details such as neurites of neurons were rendered with superior quality for image analysis as a result of boosting. For example, it was shown that neurons in scans from brains processed with vDISCO and obtained by light-sheet microscopy could be reliably traced[23] by using algorithms that were implemented for higher resolution microscopy systems such as confocal[43]. The signal from the synthetic fluorophores is also highly stable after clearing, being preserved for many months[23] and years (**Figure 2,3**). This allows repeated imaging of the same samples for a long period of time with the advantage to decrease research resources and number of animals needed in the experiments.

Besides boosting fluorescent proteins expressed by transgenic lines, vDISCO is also compatible with virus tracing or the transplantation of fluorescent cells[1]. Moreover, the use of animals expressing multiple fluorescent proteins such as GFP and RFP in specific cell populations and boosting with nanobodies conjugated with different fluorophores (e.g Atto488 and Atto647N), would allow multichannel imaging of more complex phenomena such as interaction of immune cells and the nervous system.

The vDISCO pipeline has the advantage of being simple and fast. It doesn't require special and expensive equipment and could be easily implemented by researchers from any field. In fact, it simply consists of serial incubation or perfusion of the samples with different solutions. Apart from the time needed to set up the pumping system, the actual work to change the solution takes few minutes, allowing the possibility to start multiple samples at the same time. When applied to single organs such as mouse brains, vDISCO takes about 2-3 weeks to complete the labeling and clearing with passive incubation with little handling from the operator; while whole bodies can be processed in 2-3 weeks using the active perfusion approach. Other whole body clearing methods take 11-21 days only for the clearing step[18,21,22].

Imaging and analyzing samples in 3D is also very convenient: whole organs can be imaged with the light-sheet microscope within few hours, whole bodies within 3 to 14 days depending on the imaging system, while data analysis can be concluded in 3-4 days or 1-2 weeks depending on the size of the data and the type of the analysis. These timings are definitely and significantly shorter than the ones required in standard histology: sectioning whole bodies would take several weeks, staining all the slices with antibodies additional weeks, while imaging all micrometer thick slices would take months as well as reconstructing the entire sample. Moreover, all these steps require constant handling by the operator. Automated sectioning and imaging systems such as tomographic systems exist, but they are very time-consuming, difficult to implement and therefore not routinely adopted by laboratories[44–46]. The resulting data from sectioning might present artifacts or loss of molecular and tissual details that are caused by mechanical distortions during the slicing. These problems can lead to image alignment issues and misevaluation of the data (for example the connectivity, density and topography of the objects of interest).

As last advantage, samples processed with vDISCO are compatible not only with light-sheet microscopes from different manufacturers and sources (**Figure 2b-g,3, Supplementary Figure 2**, but also with other fluorescence imaging systems such as epifluorescence (**Figure 1a**), confocal (**Figure 6a**) and 2-photon

microscopes. This versatility is useful because each imaging system has specific advantages: for example higher resolution for confocal or speed for epifluorescence.

With the above-mentioned features, vDISCO can pave the way for a new histological approach to study biological phenomena: by taking into account the entire complexity of biological systems. In fact, we are now able to comprehensively examine in details whole organisms (for example the density of cellular populations in whole bodies or the spatial distribution and extension of nerves) without being biased of selecting a particular region of interest and therefore without the risk of missing important events in other body parts. Since vDISCO was developed for whole bodies, it is virtually compatible with all organs, therefore it constitutes a universal histological technique that can be beneficial in several fields of biomedical research.

For example, vDISCO was used to reconstruct the first neuronal connectivity map[23] of a *Thy1*-GFPM mouse (line where a subset of neurons express GFP)[47]. This led to the discovery of peripheral neuronal degeneration after traumatic brain injury (TBI)[23]. Previously, the study of brain injuries mostly focused on the central nervous system (CNS), while the condition of nerves in the peripheral nervous system (PNS) after trauma was mostly ignored due to technical limitations in histology as described before.

Since vDISCO allows a global examination of biological and pathological phenomena, it was applied to holistically evaluate the extent of inflammation in different body parts after spinal cord injury[23] and, in combination with a deep-learning algorithm, to detect and quantify multi-organ metastases at single cell level in cancer mice[1].

With vDISCO, owing to the complete clearing of tissues such as bones and muscles, we could make all components of a head, including brain, skull and meninges, optically accessible. In this circumstance, vDISCO was applied to comprehensively study the infiltration of peripheral monocytes/macrophages into the brain after stroke, contributing to elucidate the routes taken by peripheral immune cells to enter into the cerebral tissue[23]. In fact, vDISCO allowed the discovery of previously unknown short skull-meninges connections between the meninges and the skull marrow that were filled with immune cells upon cerebral ischemia[23].

It is notable that vDISCO was the first whole-body clearing method that managed to clear whole mice with intact skin, while all other previous methods required the removal of this organ[16–22]. This achievement enabled the visualization of the immune cells and sensory nerves in the skin, providing information of the connectivity between the latter ones and the spinal cord[23]. This aspect of the method is particularly interesting because it can be applied to study the largest organ in a body and the interactions between this organ and other body components.

Finally vDISCO was used to boost the fluorescence signal in big mammalian intact organs, for instance with the aim of quantifying and evaluating the distribution of beta cell islets in pancreas from transgenic pigs expressing GFP[48].

Taken together, vDISCO possesses a big potential for a broad variety of studies: from basic research to translational experimentation. As mentioned before, biological organisms are not constituted by isolated and confined compartments and many diseases can affect multiple body regions. In this perspective, vDISCO can be used to elucidate how peripheral symptoms originate from brain insults or to investigate alterations in the neuronal circuitry at a big scale in neurodegenerative and psychiatric diseases. In addition, vDISCO can be applied to follow whole-body inflammatory phenomena, to assess degrees of

infections (e.g parasites), to study the cross-talk between microbiota and host, to screen stem cells or therapeutic cells (e.g CAR-T cells).

Limitations

Since vDISCO belongs to the group of organic-solvent-based clearing methods and thus consists of multiple steps characterized by the use of reagents that aim to delipidate the tissues, the maintenance of lipids and lipid-associated proteins in the tissue might be locally affected by the protocol[16,40]. In fact, it has been shown that lipophilic dyes such as DiI and myelin staining are not compatible with many clearing methods[17] including the ones using organic solvents[5], therefore they might not be detectable by the vDISCO pipeline. For the same reason, the application of vDISCO on transgenic lines expressing fluorescent proteins related to lipid-associated proteins must also be carefully evaluated, although a proper and extensive fixation of the tissue might increase the compatibility of vDISCO with these lines. In addition, the elimination of lipids might prevent the vDISCO processed samples to be further analyzed with electron microscopy[10].

By using nanobodies as anti-fluorescent proteins, vDISCO can boost up to 21 types of transgenically expressed proteins other than GFP, including mCherry, Venus etc. vDISCO is in principle compatible with nanobodies targeting endogenously expressed proteins as well[23]. However, the application of vDISCO in this perspective is limited by the lack of nanobodies developed for histology because they have been mostly optimized for *in vivo* applications, for instance as therapeutic agents or as labels for intracellular markers in living cells[49]. Therefore, our study will highly encourage the future development of new nanobodies suitable for deep tissue immunolabeling of fixed thick specimens. In this direction, the development of fluorophores belonging to the near-infrared spectrum to conjugate with the nanobodies will also further contribute to increase the imaging depth[26].

Another limitation can be represented by the microscopy system: in fact, without a dedicated microscope, clearing whole organisms would not be beneficial, simply because we would not be able to scan the sample or achieve a sufficient imaging depth. The first whole body 3D reconstruction of the nervous system from a *Thy1*-GFPM mouse was achieved using a standard light-sheet microscope (LaVision BioTec-Miltenyi Ultramicroscope II) with an imaging chamber of 8 cm x 8 cm x 3.5 cm and a sample stage travel range of 1 cm x 1 cm x 1 cm. Although organic solvent based clearing methods had the property to shrink the samples up to 65% of their original volume[16,23], the size of a whole cleared mouse (10 cm in length after shrinkage, 13-15 cm if the limbs are stretched) was still significantly bigger than the travel range of the microscope. Thus, the imaging of the intact body could not be concluded in one session, but the sample was continuously displaced or flipped to allow that all the body parts were imaged. Then the imaged volumes were stitched together[16,23]. This whole process could be quite time consuming considering the number of separated scans that were needed for one mouse, requiring about 2-3 weeks to collect 20-24 separated scans. The recent availability of light-sheet microscopes with bigger imaging chamber and sample holder stages designed for imaging large samples such as whole mice[1,48] managed to significantly reduce the time and the amount of work spent during the data acquisition session taking only 3-4 days for an adult mouse, although such systems must be coupled with very long distance objectives (minimum 20 mm) with the trade-off of reducing the resolution. Furthermore, the rate of shrinkage has to be carefully evaluated in different organs and tissues.

As last, the amount of raw data generated with our holistic approach can be difficult to handle: the scans of a single animal can occupy 2-3 terabytes[23], therefore computers with more than 256-512 gigabytes of RAM and storage systems of terabytes are needed, but they are not always available in all labs. Recent

innovations in distributed computing and cloud computing can represent interesting platforms for analysis that require high-computational power.

Moreover, data of hundreds or thousands of gigabytes are too complex or not feasible to be analyzed by a human operator. Therefore, algorithms that are able to automatically analyze the collected information are highly desired. Well known image processing software such as Fiji (ImageJ) already offer some tool[50] for automated visualization, segmentation and reconstructions of specific objects. Other computational methods for automated analysis of scans from thick tissues or cleared whole organs were published in the recent years[51]. However, all these tools are not sufficiently flexible because they mostly rely on traditional data analysis strategies: the pre-processing of the data (e.g. normalization and thresholding) is based on filters, of which parameters must be adjusted by the human operator based on the characteristics of each individual dataset. Hence, a parallel development of fully automated computational algorithms such as the ones based on deep-learning would be necessary to fully exploit the advantages of vDISCO. One of the first examples in this direction is represented by DeepMACT[1]: a highly efficient deep-learning algorithm able to detect, segment, quantify, annotate and register individual metastasis in transparent mice bearing cancer.

Experimental design: overview of the vDISCO pipeline

The general vDISCO pipeline to obtain a sample ready for imaging consists of sequential steps: (i) sample preparation, (ii) decolorization, (iii) decalcification, (iv) permeabilization and staining, (v) tissue clearing **(Figure 1)**. The decolorization and decalcification steps can be skipped if using a version of vDISCO that is based on passive incubation, which will be described later in the next paragraph.

Choice of the sample and sample preparation: whole body or single organ?

vDISCO is a robust method that has been extensively tested and reproduced in different experiments: for example on different animal lines expressing fluorescent proteins[23,48] (**Figures 3,6-8,9d-h, Supplementary Figure 2,3),** on mice transplanted with cancer cells[1] expressing fluorescent proteins (**Figure 2**) and on mice injected with viral tracers (**Figure 9a-c**). We recommend starting with samples bearing fluorescent proteins such as GFP, YFP, mCherry or RFP. To be sure about the expression, genotyping the selected animals is highly advised. In addition, note that some fluorescent proteins (e.g. tdTomato) might not be compatible with some nanobodies (see **Table 1** for compatibility information), probably due to differences in conformational structure of the protein. Preliminary tests to assess the performance of staining must be performed if the chosen transgenic line is a reporter for lipid or membrane associated proteins (e.g Myelin-Basic-Protein MBP), because vDISCO utilizes several chemicals that dissolve lipids.

Apart from whole mice, vDISCO can be applied on dissected organs such as half (**Figure 7 and Supplementary Figure 3**) or whole brains with spinal cord (**Supplementary Figure 4**), lungs, gut, adrenal glands etc. from mice as shown in **Figure 6,** on whole small pig organs such as pancreas[48], and on tissue slices as well. For these dissected organs and small samples, a simpler version of vDISCO called "**passive-vDISCO**"[23] (see **Table 1, Figures 6,7,9, Supplementary Figure 3** and the **Procedure section**) that has the decolorization and decalcification steps optional and which requires passive incubation, was used. Passive-vDISCO can be applied to quickly test and assess the performance of new nanobodies on sections or to process in an easy way individual organs or embryos. Both slices and dissected organs can be imaged with a broad variety of microscope systems including confocal, epifluorescence and light-sheet microscopes

(**Figures 6,7,9** and **Supplementary Figure 3**). Moreover, passive vDISCO can be slightly changed into a "milder" version, to overcome stability issues of certain batches of nanobodies (see **Table 1, Figure 7f-i, Supplementary Figure 3d,e** and the **Procedure section**). The decision to go with whole body vDISCO or passive-vDISCO should be made before starting the whole pipeline, since passive-vDISCO requires the removal of the specimens of interest from the body. In all vDISCO versions it is important to have samples perfused and fixed very well, because they will go through multiple chemical treatments. When the animal is euthanized and perfused with heparinized phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA), care should be taken to assess the quality of perfusion and fixation: the liver must turn from red to yellow and the specimen must become very stiff and rigid. It is also recommended to post-fix the samples for 12 (max 24) hours in 4% PFA. It is important to note that prolonged over-fixation can increase autofluorescence.

Decolorization and decalcification

Many biological components are rich of pigments, which are substances that are able to absorb light in the visible spectrum[26,27,29] and can cause autofluorescence[24,26], hence representing an obstacle in tissue clearing. Mammalian bodies possess abundance of the pigment heme contained in the blood. Therefore, we sought to find a way to remove heme from the bodies. The standard tissue preparation procedure for histology starts with the intracardial perfusion with PBS of anesthetized animals. While this system is sufficient to remove blood from organs such as brain or gut and it is generally good for neurobiological studies, we noticed that other organs such as spleen and bone marrow still retained a high amount of heme/blood by appearing dark-red[16]. Moreover, bones themselves can interfere with imaging and impede light penetration because of their calcified nature which is poor of lipids and thus difficult to clear[16,52]. To address these issues, we introduced a decolorization and a decalcification step in vDISCO, which are performed after fixing the samples, by perfusing the body with first a decolorization solution followed by a decalcification solution. These two solutions have the purpose to reduce the tissue background and increase the transparency of the body by removing the heme from the blood and the calcium from the bones. This leads to an overall increase of the quality of the detected fluorescence signal and the possibility to quantify structures beneath thick layers of tissues and bones.

The decolorization solution is made in PBS by diluting ¼ CUBIC#1[8,18], which consists of: 25 wt% urea, 25 wt% Quadrol and 15 wt% Triton X-100 in bidistilled water (dH_2O). The decolorization activity is mainly attributable to the chemical Quadrol, which belongs to the aminoalcohol compounds, a chemical family that has been previously shown to be able to efficiently elute heme from the blood[8,17–19,38]. Since CUBIC#1 was developed for clearing, the original 100% CUBIC#1 was very viscous. This great viscosity rendered CUBIC#1 not easy to be pumped by a standard peristaltic pump that was used to perfuse the bodies in the vDISCO protocol. Moreover, in our case we were interested in the decolorization properties of Quadrol, therefore we opted for a dilution of CUBIC#1. 25% was the dilution that achieved a good level of decolorization while being liquid enough to be pumped. We recommend choosing a dilution between 20-30%, that can be adjusted based on the pumping system and the amount of blood present in the body. If the pumping system works properly and the solution recirculates correctly through the cardiovascular system (from the body to the container containing the animal), the decolorization solution will slowly turn from colorless into yellow and the spleen will become from dark red to pale pink, indicating a good extraction of the heme (**Figure 5a,b**). Other strategies for the same purpose have been published before such as the use of H_2O_2 and peroxides or acid-acetone and strong bases to bleach or to dissociate the

heme, respectively. However, we strongly discourage their application here, because these chemicals are known to detrimentally affect the antigenicity of the tissue[53–55]. New reagents such as CHAPS and w/v N-Methyldiethanolamine in the SHANEL method have also been indicated as good decolorization agents and thus representing promising alternatives[48].

Since in passive-vDISCO the solutions are not delivered via perfusion and therefore the reagents can react with the sample only via diffusion (significantly slower than via the active transport system), it is possible to incubate the samples in the decolorization solution at 37°C to speed up the process (**Table 2** and **the Procedure section**).

For the decalcification step, we decided to exploit the calcium chelating properties of EDTA to remove this ion from the bones[21,22,39]. Previously, for histological preparation, acids including nitric acid or hydrochloric acid have been reported in the literature as means to decalcify the bones[56]. However, we chose EDTA because it has been already shown to be highly efficient in contributing to the clearing of bones in other clearing protocols[21,22,39] and also because the use of acids can disrupt the antigens of the tissue. The vDISCO decalcification solution consists of 10 wt/vol% EDTA dissolved in PBS. We recommend preparing the solution early in advance because this amount of EDTA in powder can take long time to dissolve. The increase of pH to basic conditions (pH 8-9) with sodium hydroxide (NaOH) can facilitate the dissolution process. Then, the decalcification solution can be pumped into the body of the animal via intracardial perfusion at room temperature for 2 days. The softening of the bones of the animal is a good indicator to assess the success of the treatment.

As said before, the decolorization and decalcification steps can be skipped when using passive-vDISCO. However, both steps can still be performed after the sample preparation and before staining if the samples are from organs rich of blood (e.g. spleen) or from bones (**Table 2**): in the first case it would be useful to passively incubate the sample for some days in the decolorization solution exchanging it every 6 hours until the solution does not turn yellow anymore, while in the latter case the sample should be incubated in the decalcification solution for some days until the calcified tissue becomes soft.

Staining and choice of the dyes/nanobodies

In whole-body vDISCO the staining step can start after the animal is decalcified and washed 2-3 times with PBS, while in passive-vDISCO, the staining can be performed straight after washing the post-fixed samples with PBS. The staining consists of two parts: permeabilization and the actual staining. In whole body vDISCO both parts are performed with active perfusion with the perfusion setting heated up to 28-30°C with an infrared lamp (**Figure 5c-i**), while in passive-vDISCO both parts are performed with passive incubation at 37°C, which is fundamental to increase the penetration of the dyes. In the permeabilization part, the samples are perfused/incubated for 1 day with a permeabilization solution containing 1.5 vol% goat serum, 0.5 vol% Triton X-100, 0.5 mM wt/vol% of methyl-β-cyclodextrin, 0.2 wt/vol% N-acetyl-L-hydroxyproline (a.k.a trans-1-acetyl-4-hydroxy-L-proline) and 0.05 wt/vol% sodium azide in PBS. Then the samples are stained by perfusion/incubation with this same solution containing the dye: 6 days of perfusion are sufficient for a whole body, while the timing for passive-vDISCO can vary depending on the size of the sample (e.g. 1-2 days for 1 mm sections, 14 days for whole mouse brains). For some batches of nanobody that show poor stability in this staining solution (**Table 1**), it is recommended to dilute the dyes in another solution made by the same concentrations of serum, Triton and sodium azide in PBS, but without methyl-β-cyclodextrin and N-acetyl-L-hydroxyproline. We called this version of vDISCO "**mild-vDISCO**" (**Table 3**).

The staining part is particularly critical especially in whole-body vDISCO: the perfusion must run flawlessly to ensure that the nanobody can reach all body districts of the animal. For this purpose, we recommend applying a drop of superglue on the perfusion needle at the level of its injection site into the heart (F**igure 4g-j**). Moreover, to avoid the accumulation of dye aggregates in the body we recommend applying a syringe filter to the ending of the injection tube (**Figure 5c-f**).

The most suitable dyes for vDISCO staining should possess a small size in order to be able to penetrate thick tissues. We found out that the small nucleus dye propidium iodide (PI) works very well to label all the nuclei of the cells of our samples with both passive-vDISCO and whole-body vDISCO[23]. Interestingly, the PI tends to accumulate in tissues dense of cells from organs such as thymus, lungs and especially bone marrow/bone, being in this way a good stain to highlight the bones of the animal. Moreover, the PI is a red dye (excitation(Ex)/emission(Em)= 535/617 , therefore it can be used in multichannel imaging together with nanobodies conjugated with far-red fluorophores: for example in the neuronal reporter line *Thy1*-GFPM, PI was used as counterstain for the bones, while the nanobody was used to follow the trajectories of the nerves through the bones (e.g. going out from the spinal cord through the vertebra)[23]. The TO-PRO-3 far-red (Ex/Em=642/661) nucleus counterstain can be used instead of PI. However, we witnessed a very big difference in performance between TO-PRO-3 batches: some batches provide very low signal (data not shown). Therefore, preliminary tests with this dye should be performed. We do not recommend DAPI (Ex/Em=350/470) as counterstain of big tissues with whole body vDISCO or passive-vDISCO, because the UV spectrum of DAPI doesn't allow the light to penetrate deep into the tissue[57], although DAPI can still be an option for staining slices.

For whole body and passive immunolabeling of the samples we exploited the small size of the nanobodies. In histology, nanobodies are mostly available as anti-fluorescent proteins: anti-GFP that can also bind (e)YFP, Venus, CFP and other GFP derivates including eGFP, sfGFP etc., or anti-RFP and derivates such as mRFP, mCherry, DsRed etc. We suggest checking the data sheet of the nanobody producer/vendor for more compatibility information: for example, the anti-RFP nanobody from the Chromotek company does not recognize tdTomato, while a particular clone from Nanotag company does, as reported in the data sheet (**Table 1**). This was confirmed in our hands while staining virus vectors expressing tdTomato in the brain (data not shown).

The nanobodies on the market are available conjugated with different fluorophores: we observed that fluorophores from the Atto and Alexa family give good performance with vDISCO (**Figures 2,3**,**6-9** and **Supplementary Figures 2,3**). One can also do a custom conjugation with the desired fluorophore (**Figure 7e, Supplementary Figure 3c**), although different chemistries for the conjugation reaction might need to be tried (NHS, maleimide, enzymatic…), depending on the sequence of the nanobody and the chosen dye: for example it is known that Atto647N is a sticky dye and if not handled properly, it can cause unspecific staining of the tissue. Moreover, when Lysine residues are present in the complementarity-determining regions (CDRs) of the nanobodies, site-directed chemistry should be favored to avoid that the labeling affects the binding capacity of the nanobody. Therefore, care should be taken during the quality control after the conjugation: purity of the conjugation, concentration, ratio dye/protein, functionality after labeling etc. If without expertise, we suggest contacting a renowned nanobody lab for the custom-conjugation.

In order to exploit the deeper penetration of long wavelength light into the tissue, we preferred to use nanobodies conjugated to far-red fluorophores such as Alexa647 or Atto647N[1,23], except when we needed to multiplex different colors (e.g. 647 nanobody anti-GFP in combination with with 594 nanobody anti-

RFP[23]). The variability of different sets of nanobodies should be carefully taken into consideration: in our experience, diverse staining performances could be observed depending on the nanobody clone, the supplier, the conjugation strategy and even on the batch of the same product (**Figure 7, Supplementary Figure 3**). For instance, whole body vDISCO was mostly performed with nanobodies from Chromotek[1,23]. These nanobodies are characterized by a high brightness and a good penetration capability in whole bodies[1,23], but in the standard passive-vDISCO protocol (**Figure 7a-e, Supplementary Figure 3a-c**) they showed variable results in terms of stability between batches (e.g. **Figure 7a** vs. **7b**), indicating a possible issue in the chemical bond between the nanobody and the fluorophore. Therefore, some batches from Chromotek require the use of a milder version of vDISCO mentioned before (**Table 1**, **Figure 7f-i, Supplementary Figure 3d,e** and **procedure section**). Nanobodies against fluorescent proteins manufactured at the Nanotag company were tested for vDISCO passive staining. For example, we tested: FluoTag-X4 made by two clones that recognize two distinct epitopes of the same protein and each clone is coupled with two fluorophores (**Table 1, Figure 7c, Supplementary Figure 3a**), and FluoTag-Q (**Table 1, Figure 7d,9d-h, Supplementary Figure 3b,**) made by a clone coupled exactly 1 to 1 with the fluorophore molecules. Fluotag-X4 provided high brightness but lacked penetration power (e.g. **Figure 7c** vs. **7i** and **Supplementary Figure 3a vs. 3e**), on the other hand, FluoTag-Q gave excellent results for passive staining of microglia and pericytes expressing GFP (**Figure 7d,9d-h**), although also showing less penetration power than the Chromotek nanobodies in the *Thy1*-GFPM line **(Supplementary Figure 3b vs 3e**)**.** In general, Nanotag nanobodies demonstrated very high stability, without the need to use mild-vDISCO (**Table1**, **Figure 7**). Since the nanobody clones are different from company to company, we hypothesize that the different penetration ability might be due to the surface charges of the clones. Nanotag nanobodies possess more negative charges on the surface that might interact with the tissue, slowing the penetration, while Chromotek nanobodies tend to have an almost neutral charge on the surface (personal communication). Other intrinsic properties of nanobodies can affect the overall penetration capability too, such as: distribution of charges in the amino-acid sequence, their CDRs, the affinity and the epitope recognized. Nevertheless, the penetration performance can be increased by extending the incubation times for passive staining (about 20-30% longer) or/and increasing the temperature up to 40°C. We also tested a custom-made nanobody conjugated to Alexa 647 with passive-vDISCO, which provided good staining in terms of signal stability, penetration and brightness for the neuronal reporter line *Thy1*-GFPM (**Supplementary Figure 3c**). Regarding the concentration, 0.08–0.14 µg ml^{-1} of nanobody is sufficient to stain a whole mouse, while 10x more concentrated nanobody is required for whole mouse brains processed with passive-vDISCO (**Table 1**). However, in both cases the concentration can be adjusted based on the expression of the fluorescent protein and the size of the sample.

Last, it is highly recommended that the chosen nanobodies should be validated before starting the experiments. The validation has the purpose to disclose whether the nanobody is able to sustain the staining and clearing conditions used in vDISCO. The validation of a new nanobody can be performed using passive-vDISCO (see **Materials and Procedure parts**). In general, nanobodies that do not normally give a good performance in standard IHC should not be used with vDISCO. For more information about the tested samples, compatible fluorescent proteins, suggested concentrations, batch/lot numbers, suggested protocol, performances of staining and other information see **Table 1**.

Tissue clearing

After staining, the samples are ready to be cleared. For the clearing process we decided to use a slightly modified version of 3DISCO[5,40] because we found out that this protocol is highly reproducible, very simple,

fast, can be performed at room temperature, doesn't require special setting like a perfusion system and as all organic solvent based clearing methods, it can achieve a high level of transparency[31]. Given the advantages, this method is strongly convenient for whole body clearing, when the good transparency is much required in a thick sample. The clearing procedure is performed exactly in the same way for both whole bodies and dissected samples: through passive incubations with organic solvents. Whole mice or big organs such as pig pancreas[48] can be placed in glass containers that can hold at least 350 ml while slices or small mouse organs can be placed in plastic tubes or glass jars. Since the organic solvents can often melt plastic, it is very important to test if the plastic containers that are planned to be used are sufficiently resistant. From our experience, polypropylene from specific manufacturers stands organic solvents well: 50 ml tubes from Falcon brand and 5 ml tubes from Eppendorf brand showed good resistance to the clearing chemicals. 15ml tubes from Falcon brand are not recommended, because long incubations with chemicals tend to break the lids of these tubes.

Overall, the clearing protocol is straightforward because it is based on the sequential passive incubation of the specimen in organic solvents: an ascending series of 4 dilutions (50, 70, 80, 100 vol% in bidistilled water) of THF to dehydrate, DCM to delipidate and BABB (which was replaced by DBE in the original 3DISCO) to reach the transparency. The clearing procedure requires the simple exchange of one solution with the next one and the action of replacing the solution takes just a couple of minutes for each sample, no matter the size. The duration of each incubation is highly flexible and depends on the size of the sample and its lipid content. For example a small specimen such a brain slice or a mouse adrenal gland would require about 45-60 minutes of incubation in each of the THF solutions, a whole brain (rich of white matter/lipids) 2 hours (with the last 100% THF step extended to 12 hours/overnight), a whole body 12 hours (**Table 2,3**). The following delipidation with DCM is optional and it is recommended if dealing with lipid rich organs or whole bodies. Generally, DCM treatment takes 15 minutes for small samples including tissue pieces and slices, 1-2 hours for whole brains, 3 hours for whole bodies (**Table 2,4**). Timings (**Table 2,4**) can be adjusted and extended based on the preliminary tests. In case of working with overweight animals, we suggest to prolong all the steps of clearing.

Besides, the flexibility of this clearing method is also due to the fact that the fluorophores used in vDISCO are highly stable in the clearing reagents[23]. Although we do not suggest over-incubating the samples for multiple days in the clearing solutions (except for the BABB which works as storing solution as well), we did not observe problems in detecting the fluorescence signal when the samples were accidentally left for many days in the chemicals. Naturally, since the whole clearing requires just few minutes for handling the samples, one can clear simultaneously many samples or can dedicate the time to other experiments, while waiting for the incubations. The transparency is achieved after placing the sample in the RI matching solution BABB: there is a minimum incubation time to achieve the transparency (e.g. 2 hours for a brain slice, 12 hours for a whole brain and 1 day for a whole mouse). The level of transparency will likely increase over time as more RI matching solution penetrates into the sample and should be visually assessed before imaging. BABB can be replaced with DBE with comparable results, although we chose to use BABB because it is slightly denser than DBE therefore samples are less subjected to moving during imaging. The change of color of the sample which turns yellow after clearing is not an issue for imaging. Leaving the samples in BABB does not represent a problem since vDISCO allows repetitive imaging in months or years[23] (**Figure 2,3**). We suggest storing the samples protected from light and at room temperature, although we did not experience fading of the signal if stored at 4°C. Despite the simplicity of the whole procedure, care should be taken while handling the solvents, performing the protocol and disposing the wastes, because some of

the solvents possess moderate toxicity and are hazardous for the operator and/or the environment (see **Box 1** for further information).

Last, we observed that samples stained with vDISCO can be cleared with other clearing protocols such as iDISCO+ based on methanol dehydration (data not shown). This finding is interesting if the advantages of other clearing methods are desired: for example iDISCO+ clearing is known to shrink the sample in less extent compared with 3DISCO[51].

Imaging

The vDISCO processed samples can be imaged with a variety of imaging systems. It is important to mention that the samples will stay transparent as long as they are completely submerged in the RI matching solution (BABB), thus this solution must constitute the sample mounting medium for imaging as well.

The choice of the objective is also fundamental to produce high quality data. Ideally the objectives should provide decent resolution with as high as possible numerical aperture (NA) but at the same time they should have long working-distance (WD), able to cover the whole thickness of the sample. The objectives can be air lenses or immersion lenses, the latter ones should be optimized for the RI of the RI matching solution or of the immersion medium, in order to reduce optical aberrations and increase the resolution.

To quickly assess if the staining was successful (e.g. testing slices) or to get whole body 2D images in 10-15 minutes, it is possible to use an epifluorescence microscope such as the Zeiss AxioZoom EMS3/SyCoP3 (**Supplementary Figure 5a-d**), which can support a long WD air objective (PlanZ x1, 0.25 NA, WD = 56 mm)[1,23] .

On the other hand, to achieve 3D high resolution imaging we used light-sheet microscopes[15] (**Supplementary Figure 6,7**). Light-sheet systems are ideal for cleared samples because they illuminate the sample with a sheet of light: in this way the whole illuminated focal plane will be simultaneously captured with a scientific complementary metal oxide semiconductor (sCMOS) camera, allowing very fast imaging and low photobleaching.

In particular, we used LaVision BioTec-Miltenyi Ultramicroscope II, LaVision BioTec-Miltenyi Blaze microscope for large samples, Zeiss Lightsheet Z.1 and mesoSPIM.

The Ultramicroscope II has an imaging chamber of 8 x 8 x 3.5 cm with a standard sample holder travel range of 1 x 1 x 1 cm (in the xyz axes) which we increased to 1.1 cm in z by a custom-made stage. This microscope was used to create the first whole body neuronal map of a *Thy1*-GFPM mouse[23], by using a 1x air objective (Olympus MV PLAPO ×1, 0.25 NA, WD = 65 mm) coupled with a zoom body kept at 0.63x. With these settings the field of view (FOV) was 2 x 2.5 cm, which was able to cover the entire width of a mouse body. However, since the travel range of the sample holder was smaller than the size of the entire body volume, multiple scans of different body parts were first taken by displacing the body after each scan, and then the scans were stitched all together. For labs owning the Ultramicroscope II we suggest this strategy to image whole mice or samples bigger than the travel range, keeping in mind that the process of mounting the sample can be quite complicated and the entire imaging step can take 1-2 weeks (**Supplementary Figure 6a-j**).

Therefore, we suggest using light-sheet microscopes with bigger sample holders and imaging chambers such as the LaVision Biotec-Miltenyi Blaze light-sheet microscope which is optimized for large samples, including whole bodies[1] and intact human kidneys[48]. Our Blaze system possesses an elongated customized sample holder which can travel 4 x 9 x 5 cm in the xyz axes and a large imaging chamber of 25 x 7 x 9 cm. Moreover, it works with a dipping 1.1x objective (MI PLAN x1.1, 0.1 NA, WD = 17 mm) which has a FOV of

1.2 x 1.2 cm. With this microscope an entire mouse can be imaged with a single tiling scan just in 2-3 days, although we recommend flipping the body up-side down after the first scan to achieve the best resolution from both sides.

The Zeiss Lightsheet Z.1 is a light-sheet microscope with a closed chamber optimized for single organs or small organisms imaging (**Supplementary Figure 7**). In fact it has an imaging chamber of 1 x 1 x 2 cm, a sample holder travel range of 1 x 5 x 1 cm and a rotation stage for multi-angle scans allowing for isotropic resolution in 3D (**Figure 3**).

We used the Lightsheet Z.1 and the Ultramicrope II to capture scans of whole mouse organs (e.g lungs, heart, brain) with higher magnification and NA objectives such as a 5x objective (Zeiss EC Plan-Neofluar 5x/0,16 NA, WD = 10.5 mm) (**Figure 3**), a 4x objective (Olympus XLFLUOR4x/340 ×4 corrected, 0.28 NA, WD = 10 mm) (**Figure 6b-f,7,9d-h, Supplementary Figure 3**) or a 20x objective (Zeiss Clr Plan-Neofluar, 20x/1.0 NA, WD 5.6 = mm)[23] (**Figure 9a-c**).

Cleared samples can subsequently be dissected from the vDISCO processed body in order to be imaged with higher resolution but slower microscopes including confocal[1,23] (**Supplementary Figure 5e-g**) and 2-photon microscopes, being aware that the commercial systems of these microscopes are normally coupled with high NA objectives therefore their WD are limited. However, the confocal and 2-photon microscopes can be good alternatives for labs lacking light-sheet systems. In addition, they provide the advantage of being able to look at specific regions in more details after a whole-body screen with light-sheet microscopy. Here, samples processed with vDISCO were imaged with the laser scanning confocal microscope Zeiss LSM 880 by using a long WD 25x objective (Leica, 0.95 NA, WD = 2.5 mm) mounted on a custom mounting thread[23] (**Figures 6a, 8j, 9h**) . We recommend the users to choose the objective based on their applications and if possible, ask to the microscope manufacturer for eventual customizations.

We also suggest capturing images with a pixel intensity range of 16 bits in order to resolve details in images with high contrast in intensity. Regarding the light-sheet systems, we also recommend selecting the thinnest sheet available and setting the Z-step interval according to the size of the structures of interest (e.g 2-10 µm for single cell resolution).

Last, the mesoSPIM[58] light-sheet microscope is a promising option for imaging large transparent samples. This microscope is characterized by an isotropic resolution and can achieve very high scanning speeds (15 minutes per mouse brain at 5-µm sampling vs. 3 hours of the Ultramicroscpe II, thus 12x faster), owing also to the FOV of 2-20 mm, the travel range of 52 mm × 52 mm × 102 mm and the 360° rotating sample holder for multiview imaging[58]. Owing to its large travel range, a mesoSPIM is capable of imaging the whole mouse CNS[58] without remounting the sample. Since the mesoSPIM is fully customizable, a version tailored for imaging whole mice was built by updating the published version. This new mesoSPIM possesses the features for obtaining high quality result for whole body imaging (**Supplementary Figure 2**), since it has a large (100 ×200 ×100 mm) XYZ travel range, it uses imaging cuvettes of 30 x 30 x 120 mm or 40 x 40 x 120 mm and it has an easy strategy to mount the samples based on self-centering magnets (**Supplementary Figure 7**).

Regarding the data collection, raw images are collected as grayscale TIFF stacks, or scans are saved as Zeiss CZI files if from Zeiss microscopes. In the latter case the single images constituting a stack can then exported as TIFF files as well.

Data processing and analysis

With the development of vDISCO, we have provided a technology that allows scientists to comprehensively and quantitatively study biological phenomena in whole organs and whole bodies, paving the way, for example, for the realization of whole body connectome maps. This holistic approach, as expected, generates an incredible amount of data from hundreds of gigabytes for individual organs to some terabytes for a single mouse. These data not only require adequate storing and processing strategies, but they are also extremely complex, containing a vast diversity of biological information. How to handle these massive data, how to analyze them and how to get scientifically meaningful information from them?

First, the size of the data can be reduced by simply compressing the raw TIFF files with a lossless compression algorithm: we suggest Lempel-Ziv-Welch (LZW) which is widely supported among common scientific software by default. Some commercial software for image rendering and analysis might have their own compression format (e.g. HDF5 in the IMS format from Bitplane Imaris or the SIS format of Arivis Vision4D), generally we convert our stacks to these formats for a fast loading of the files and keeping a copy of the raw data at the same time.

Almost all our data were generated as tiling scans, which were performed by either manually displacing the sample during imaging (e.g. AxioZoom) or by exploiting the motorized XYZ stages of the microscopes. Tiling scanning is crucial when imaging large volumes at subcellular resolution. Therefore, after imaging, a stitching step is needed to reconstruct the final image. 2D image tiles generated by AxioZoom were manually aligned in the XY axes with each other based on visual inspection and merged together with Adobe Photoshop CS6 using its "Photomerge" function. Tiling scans saved in CZI format were automatically stitched by the Zen (Zeiss) acquisition software immediately after completing the acquisition; while tiling scans saved as TIFF stacks were stitched using Fiji[50] (ImageJ)´s stitching plugin[59] (**Supplementary Figure 8**); optionally the removal of acquisition errors/shiftings can be done by using Fiji's TrakEM2 plugin and Imglib2library. Alternatively, tiling errors in the XY and Z dimensions can be corrected using TeraStitcher[60] (v.1.10; https://abria.github.io/TeraStitcher/). It is noteworthy to mention that the whole mouse stitching/reconstruction can be heavily impaired by insufficient RAM. However, this can be overcome using read on demand and caching implementations/code as we did in Volume Fusion module of the Vision4D (Arivis) to stitch the whole mouse scan (**Supplementary Figure 9,10**).

Data visualization and simple processing including filtering, equalization, histogram adjustments and contrast enhancement can be performed with several softwares: Fiji, Amira (FEI Visualization Sciences Group), Imaris (Bitplane) and Vision4D, the last three ones are also used for 3D rendering of the scans. In particular, we appreciated Fiji for being user-friendly and open-source and we used it for fluorescent signal characterizations, while Imaris was applied to produce most of the 3D volume renderings and videos. All the above-mentioned software packages include tools for segmentation, tracing, quantification and manual annotation. For more specialized applications, one can also use software such as Neurolucida[61] or NeuroGPS-Tree[43] to analyze the morphology and quantify the features of the neurites[23] in neurons, or ClearMap[51] to automatically count, register and annotate cells with distinguishable round soma to the Allen Mouse Brain Atlas. All mentioned software come with a diverse degree of automation in analysis: for instance one can either manually segment all the cell processes in a scan with the selection tools in Fiji or rely on the automated segmentation function of NeuroGPS-Tree. However, all the computer programs listed so far have limitations that should be considered: first the data size that they can handle is determined either by the RAM of the computer or by an intrinsic characteristic of the software: for example NeuroGPS-Tree can only run data smaller than 1 gigabyte, therefore it requires a down sampling step during the pre-processing (jeopardizing in this way the details of biological structures). Second,

although some of these tools can perform automated analysis, all of them rely on traditional analytical approaches based on explicit pre-processing and filter-based recognition. In fact, the parameters needed for the analysis must be adjusted and specified by a human operator, who will tailor each specific dataset based on its characteristics. Since fully automated data analysis is greatly desired due to the complexity and the amount of data produced by vDISCO pipeline, new computer tools based on artificial intelligence (AI) have now started solving these bottlenecks. In fact, the high contrasted images that vDISCO is able to provide, represent good data that can be analyzed using deep learning algorithms. These algorithms are able to learn from information provided by human experts and subsequently they can dynamically and autonomously adjust their criteria of analysis depending on the characteristics of the new dataset[62,63]. In this way, deep learning algorithms can reliably process large information in a very fast and accurate manner without the supervision of a human operator[64], and even outperforming human experts[65]. For instance, we applied deep learning on vDISCO cleared animals to automatically detect single metastasis in intact mice of cancer models[1].

Computational power, data storing and backing up

As said, the amount of data that can be produced using vDISCO pipeline can span from hundreds of gigabytes to terabyte. It is not surprising that the analysis of such an amount of data can represent the biggest burden and the most-time consuming step of the whole pipeline, if without adequate computer systems.

For our analysis we used different standalone workstations: a HP Z820 with 196 GB RAM and with 8 core Xeon processor and nVidia Quadro k5000 graphics card, a HP Z840 dual Xeon 256 GB DDR4 RAM and with nVidia Quadro M5000 8GB graphic card, a HP Z840 with 512 GB RAM and with Intel Xeon E5-2640 v4 @ 2.40GHz x 40 combined with a Quadro P5000 graphic card. All workstations can support Windows and Linux operating systems (OS) in dual boot mode. Windows can be used for standard or commercially available software, while Linux is the ideal OS for machine learning and deep learning algorithm coding, since many frameworks developed for this purpose were optimized for Linux. If purchasing a new computer, we recommend choosing one with at least 256 GB of RAM and at least one SSD drive for the operating system. In addition, we use multiple Promise Technology Pegasus2NAS (network attached storage with 96 TB capacity) devices to store the daily collected data and a couple of Synology RS2418 NAS (with 200 TB capacity) devices as backup and long-term storage system on 10-Gbit/s-Ethernet layout.

A promising option to obtain high computational power is to adopt cloud computing, which recently has been seen rising in private sectors and in some labs[66]. In small labs, it is possible to start with an implementation of this strategy using local workstations. As the need for computing power arises with more data, another alternative is to migrate the workload to Cloud platforms such as Digital Ocean, Amazon Web Services (AWS), Google Cloud Platform (GCP) and Microsoft Azure, where a cluster of high-performing interconnected computers can run data-crunching software in a distributed manner.

Finally, we suggest that the data from the microscope should not be collected directly in a network drive while acquiring the image, because a failure of the connection in the network would jeopardize the whole scan. Rather, data should be stored on a hard drive with a RAID configuration for speed and redundancy (depending on implemented local policy).

Materials

Reagents

Samples

Samples come from animals expressing fluorescent proteins that have been shown to be recognized by a particular nanobody according to the nanobody manufacturer website. The expression of the fluorescent protein must be confirmed by genotyping prior commencing the experiment. The sample can express the fluorescent protein also with viral strategies, alternatively the sample can come from animals transplanted or injected with fluorescent protein expressing cells. The following mouse lines are examples of suitable strains to use: *Thy1*-GFPM[47] (or *Thy1*-YFPH), CX3CR1$^{GFP/+}$ (B6.129P-Cx3cr1tm1Litt/J; Jackson Laboratory strain code 005582[36]), *Prox1*-EGFP (Tg(Prox1-EGFP)KY221Gsat/Mmucd; Mutant Mouse Resource and Research Centers strain code 031006-UCD) and PDGFRb-EGFP (Mouse Genome Informatics strain code 4847307) are shown in this study; *LySM*-GFP (Lyz2tm1.1^{Graf}, Mouse Genome Informatics strain code 2654931), CCR2$^{RFP/+}$ (B6.129(Cg)-Ccr2tm2.1Ifc/J; Jackson Laboratory strain code 017586) and *CD68*-GFP (C57BL/6-Tg(CD68-EGFP)1Drg/j; Jackson Laboratory strain code 026827) were published before[23]. Other examples used here are: a C57BL/6 mouse transplanted with murine syngeneic R254 pancreatic cancer cells expressing eGFP for 38 days, an adult Emx1-Cre x RΦGT mice[67,68] injected with EnvA-pseudotyped G-deleted rabies virus expressing GFP (SADB19[69]) in the neocortex.

CRITICAL Animal experimentation must be in accordance with all the relevant governmental and institutional regulations. The animal housing and experiments in this work were conducted in conform to the institutional guidelines (Klinikum der Universität München/Ludwig Maximilian University of Munich, Technische Universitaet Muenchen, Regierung von Oberbayern and UK Home Office), after approval of the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany), and in accordance with the European directive 2010/63/EU for animal research.

Reagents for sample preparation

- 10x Phosphate-buffered Saline (PBS) (0.1M, stock solution, Apotheke Klinikum der Universität Munchen, cat. no. P32799) **CRITICAL** PBS can come from a variety of suppliers. Check with the supplier the modality of preparation
- Bidistilled water (dH_2O)
- MMF triple combination anesthetics: midazolam, medetomidine and fentanyl (1 ml per 100 g body mass for mice; intraperitoneal) **CRITICAL** follow the regulations of your institution regarding the drugs used for anesthesia
- Heparin (5000U/ml, Ratiopharm, cat. no N68542.03)
- 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 in dark and use it within expiration period only
- Sodium azide (Sigma-Aldrich, cat. no. 71290) **CAUTION** Very toxic reagent

Reagent for decolorization, decalcification and immunostaining

- Urea (Carl Roth, cat. no. 3941.3)
- 10x PBS
- dH_2O

- Quadrol a.k.a. N,N,N',N'-tetrakis (2-hydroxypropyl)ethylenediamine (Sigma-Aldrich, cat. no. 122262)
- Triton X-100 (AppliChem, cat. no. A4975,1000)
- EDTA (Carl Roth, cat. no. 8040)
- NaOH (Sigma-Aldrich, cat. no. 71687)
- Goat serum (Gibco, cat. no. 16210072)
- Methyl-beta-cyclodextrin (Sigma, cat. no. 332615)
- *trans*-1-Acetyl-4-hydroxy-L-proline a.k.a *N*-acetyl-L-hydroxyproline (Sigma-Aldrich, cat. no. 441562)
- Propidium Iodide (PI, Sigma-Aldrich, cat. no. P4864 or Invitrogen cat. no. P3566)
- nanobody: see **Table 1**

Reagents for clearing

- THF (Sigma-Aldrich, cat. no. 186562)
- DCM (Sigma-Aldrich, cat. no. 270997)
- Benzyl alcohol (Sigma-Aldrich, cat. no. 24122)
- Benzyl benzoate (Sigma-Aldrich, cat. no. W213802)

Equipment

General equipment and supplies

- Perfusion One system (Leica)
- Disposable 30 ml syringes (any)
- Disposable 1 ml syringes (Braun, cat. no. 9166017V)
- Disposable 1 ml syringes with 25G needle (Braun, model Injekt-F, cat. no. 9166033V)
- Micro-Fine Ultra needles 12,7mm 0,33 mm 29G (BD)
- Microlance 3 needles 0.3mm, 13mm 30G (BD, cat. no. 304000)
- Surgery scissors (FST, cat. no. 14958-11)
- Large surgical tweezers (FST, cat. no. 11000-20)
- Fine surgical tweezers (FST, cat. no. 11252-40)
- Razor blade (Personna, cat. no. 604305-001001)
- Hair removal cream (optional, i.e. Veet)
- 5 ml tubes (Eppendorf, cat. no. 0030 119.401) **CRITICAL** we recommend these particular tubes because they are resistant to the clearing solutions
- 50 ml tubes (Thermo Fisher Scientific, cat. no. 339653) **CRITICAL** we recommend these particular tubes because they are resistant to the clearing solutions. Tubes from Falcon brand (cat. no. 352070) are also resistant to the clearing solutions, although BABB tends to leak out from the interface between lid and tube
- 24 multiwell (Falcon, cat. no. 353504) **CRITICAL** multiwell and cell culture plastic is generally **NOT** resistant to the clearing solutions. These containers can be used for all the steps before clearing, but not for clearing
- 35 mm glass-bottom petri dishes (MatTek, cat. no. P35G-0-14-C)

- 5 ml disposable Pasteur plastic pipettes (Alpha laboratories, cat. no. LW4728). They are resistant to the clearing solutions
- Pipette boy (any, we used the one from Roth, model accu-jet pro, cat. no. NA55.1)
- 50 ml serological pipettes (Corning Costar Stripette, cat. no. CORN4490) **CRITICAL** not resistant to the clearing solutions
- 10 or 25 serological pipettes (Corning Costar Stripette) **CRITICAL** not resistant to the clearing solutions
- 300 ml glass chamber with lid (Omnilab, cat. no. 5163279)
- Glass jars for histological staining with lids (any, we used the ones from vwr: staining jar, Hellendahl, with enlargement, cat. no. 631-0698)
- Glass petri dishes (any)
- 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)
- 1 Büchner flask or vacuum glass flask with a rubber stopper (any) **OPTIONAL**
- PFTE covered magnetic stirring bars (any)
- Hot magnetic stirrer (IKA, model RCT basic B-5000, cat No. 0003810000)
- Aluminum foil (any)
- Parafilm (Bemis, cat. no. PM-992)
- Transparent sticky tape (any)
- Black sticky tape (any)
- Liquid Superglue (Pattex, cat. no. PSK1C or Toolcraft, Superglue rapid 200, cat. no. 886515)
- Maxi-Cure Super Glue, CA (Bob Smith Industries, cat. no. BSI-113)
- Insta-Set, CA Accelerator (Bob Smith Industries, cat. no. BSI-152)
- Kleenex or adsorbent kitchen paper (any)
- White precision wipes (Kimtech Science, cat no. 05511)
- Permanent ink labelling pens (any)
- Any sample and tube holders with the shape as in **Figure 4a**
- 0.22 µm syringe filters (Sartorius, cat. no. 16532)
- Perfusion needle (Leica, cat. no. 39471024)
- Peristaltic pump (ISMATEC, REGLO Digital MS-4/8 ISM 834)
- Reference tubing for the ISMATEC peristaltic pump (ISMATEC, cat. no. SC0266)
- Hose tubing connectors for 3-5mm diameter (Omnilab, cat. no. 5434482 or 8700-0406)
- PVC tubing (Omnilab, cat. no. 5437920)
- Infrared lamp (Beuer, cat. no. IL21)
- pH meter (WTW, model pH7110)
- Vibratome (Leica, model VT1200S)
- Incubator (Memmert, model UN160), although any incubator that keeps 37°C with a shaker inside is fine
- Cordless multi-tool with a thin rotary blade (0.2 mm; Dremel, model 8200)

- Shaking rocker (IKA, model 2D digital)
- Shaker (IKA, model KS 260 basic)
- Fume hood

Imaging systems

Any fluorescence imaging system is suitable; data here were taken with the following microscopes:

Zeiss AxioZoom EMS3/SyCoP3 fluorescence stereomicroscope with:

- ×1 air objective (Plan Z ×1, 0.25 NA, WD = 56 mm)

LaVision BioTec-Miltenyi Ultramicroscope II light-sheet microscope with:

- 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
- Andor sCMOS camera Neo 5.5 (Andor, mod. no DC-152Q-C00-FI)
- SuperK EXTREME/FIANIUM supercontinuum white light laser (NKT Photonics, model SuperK EXTREME EXW-12)
- 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 XLFLUOR340 ×4 corrected/0.28 NA, WD = 10 mm),
- 20x immersion objective (Zeiss ×20 Clr Plan-Neofluar/1.0 NA, WD 5.6 = mm)

LaVision Biotec-Miltenyi Ultramicroscope Blaze light-sheet microscope with:

- 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
- sCMOS camera 4.2 Megapixel
- LaVision laser beam combiner with laser lines 488,561,639
- Single arm sample holder (**Supplementary Figure 6 k-r**)
- Customized large imaging chamber (25 x 7 x 9 cm Len-Wid-Height)
- 1.1x objective (LaVision BioTec-Miltenyi MI PLAN x1.1/0.1 NA, WD = 17 mm)
- 12x objective (LaVision BioTec-Miltenyi MI PLAN x12/0.53 NA, WD = 10 mm with dipping cap for organic solvents)

Zeiss Lightsheet Z.1 light-sheet microscope:

- Detection objective: Zeiss x5/0.16 NA air objective compatible with water-based and clearing solutions n=1.45)
- Illumination: Zeiss LSFM x5/0.1 NA objectives (for dual side illumination), illumination in Pivot mode for stripe reduction
- Cameras: two pco.edge 4.2 sCMOS cameras

- Filter set: 1) SBS LP 490, EF BP 420-470, EF BP 505-545 2) SBS LP, 560 EF BP 505-545, EF LP 660 3) SBS LP 510, EF BP 420-470, EF BP 575-615 4) SBS LP 560, EF BP 505-545, EF BP 575-615 5) SBS LP 560, EF BP 505-545, EF LP 585 6) SBS LP 640, EF BP 575-615, EF LP 660
- Chamber: 5x Clearing chamber for sample size of 1x1x2 cm
- Excitation lasers: 405 nm, 445 nm, 488 nm, 515 nm, 561 nm, 638 nm

Modified mesoSPIM light-sheet microscope with:

- Omicron SOLE-6 laser combiner with 405 nm, 488 nm, 515 nm, 561 nm, 594 nm, and 647 nm laser lines and two output fibers
- AHF QuadLine Rejectionband ZET405/488/561/640 emission filter with 50 mm diameter
- Sample XYZ & rotation stages composed of two Steinmayer Mechatronik PMT-160-DC stages with 100 and 200 mm travel range for XY-movements and a combination of Physik Instrumente M-406.4PD and M-061.PD stages for Z-movements and rotation, respectively
- Physik Instrumente M-605 stage for focusing the detection path
- Edmund Optics F-Mount PlatinumTL Telecentric lens with 0.9x magnification and lens clamp
- Teledyne Photometrics Iris 15 camera with 15 megapixel resolution
- Optomechanical architecture and electronic controlling system of mesoSPIM (see https://github.com/mesoSPIM/mesoSPIM-hardware-documentation)
- Custom 40 mm × 40 mm x 120 mm cuvette for mounting samples (Portmann Instruments)
- Magnetic holder for 40 × 40 cuvettes (https://github.com/mesoSPIM/mesoSPIM-hardware-documentation/blob/master/mesoSPIM_V5/drawings/Large-cuvette-mount-40mm-V3.pdf)
- mesoSPIM software (https://github.com/mesoSPIM/mesoSPIM-control)

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 with the objectives from different other brands

Data processing and analysis tools/software

- Vision4D (v.3.0.1 ×64, Arivis)
- Arivis converter (v.2.12.6, Arivis)
- Amira (v.6.3.0, FEI Visualization Sciences Group)
- Imaris (v.9.1, Bitplane)
- Fiji[57] (ImageJ2, v.1.51, https://fiji.sc/)
- TeraStitcher[60] (v.1.10, https://abria.github.io/TeraStitcher/) **OPTIONAL**
- Total Commander (v. 8.52a x64, https://www.ghisler.com/) **OPTIONAL**
- Photoshop CS6 (v. 13.0, Adobe)

Reagent set up

CAUTION All the reagents prepared for vDISCO must be discarded according to institutional regulations. All personnel must have adequate safety training and equipment (lab coat, safety goggles, fume hood,

etc…) for working with hazardous (flammable, toxic, volatile, irritant, environmentally harmful) materials. Considerations about clearing reagents toxicity can be found in **Box 1**

1x (0.01M) PBS

Dilute ten times 10x PBS with dH_2O to obtain 1x PBS. 1x PBS solution can be stored at room temperature (18–25 °C) for several months.

Heparinized PBS

Dilute the stock solution of heparin (5000U/ml) into 1 L of 1X PBS to reach final concentration of heparin 25U/ml. This solution is used during the perfusion for the sample preparation step to help flush the blood out of the body. This solution can be kept for some weeks at room temperature.

4 wt/vol% PFA

This solution is used as fixative solution for the perfusion and the post-fixation of the animal during the sample preparation step. We purchase 4% pH 7.4 PFA ready-to-use solution directly from the supplier for convenience. Otherwise, any 4% PFA in 1x PBS for perfusion is usable. Our PFA, if stored at 4°C, lasts until the date indicated by the supplier. When needed and if using the Leica perfusion system to perfuse the aniamls, pour it into one of the tanks of the Leica perfusion system before starting the sample preparation step at room temperature. **CAUTION** Toxic reagent. Avoid inhalation or contact with skin and eyes. Handle it in a fume hood.

PBS + Sodium Azide

Dissolve the sodium azide in powder into 1x PBS reaching a final concentration of 0.05 wt/vol%. This solution is used as storing solution for unprocessed samples obtained from the sample preparation step. This solution can be stored at room temperature for several months. **CAUTION** This solution is toxic. Avoid contact with skin and eyes.

Decolorization solution

Stock solution preparation: in a big beaker mix 25 wt% urea, 25 wt% Quadrol and 15 wt% Triton X-100 in dH_2O. To facilitate the dissolution, we suggest stirring with a magnetic stirring bar while heating up the stirrer up to 60°C. While stirring, cover the beaker with aluminum foil to prevent water evaporation. When the solution turns completely transparent and with no aggregates inside, pour it in a glass bottle and let it cool down at room temperature. The stock solution can be stored for 2-3 months at room temperature. We recommend preparing 1-2 L of the stock. **CRITICAL** Avoid boiling the stock solution when stirring. Owing to the high amount of detergent, be careful of not generating too many bubbles or foam when mixing.

Final decolorization solution preparation: dilute the stock solution 20-30 vol/vol% with 1x PBS (for whole body active vDISCO use 25%). Mix well until the final solution turns homogenous and transparent. 1 L should be enough to process one animal with whole-body vDISCO. It is optional to use this solution in passive-vDISCO (see procedure part, passive-vDISCO, decolorization).

Decalcification solution

Dissolve 10 wt/vol% EDTA in 1x PBS by stirring with a magnetic stirring bar and by adding NaOH in powder until the final solution reaches about pH=8. The NaOH is necessary to help the EDTA dissolve. Heating the solution is not recommended, because the reaction will already generate heat and the pH should be measured when the solution has already cooled down. The solution will be ready to use when the EDTA has completely dissolved, leaving no visible aggregates in the solution. 1 L of solution is enough for whole-body vDISCO of 3-4 animals. The decalcification solution can be stored at 4°C for several months. This solution can be skipped if applying passive-vDISCO on not calcified tissues (see procedure part, passive-vDISCO, decalcification).

Permeabilization/staining solution

The permeabilization/staining solution is used in both permeabilization step and staining step (it contains the dyes in the staining step). Prepare it by mixing 1.5 vol/vol% goat serum, 0.5 vol/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 1x PBS. 600-700 ml is enough to process one animal with whole body vDISCO. **CRITICAL** Prepare fresh solution before starting the experiment.

Mild staining/washing solution

The mild staining/washing solution is used as washing solution in standard vDISCO, while it is used as both staining solution and washing solution in mild-vDISCO. Prepare it by mixing 1.5 vol/vol% goat serum, 0.5 vol/vol% Triton X-100 and 0.05 wt/vol% sodium azide in 1x PBS. 600-700 ml is enough to process one animal with whole body vDISCO. **CRITICAL** Prepare fresh solution before starting the experiment.

Dehydration solutions

Transfer 500 ml (this amount might be decreased to 100 ml for small samples) of pure 100% THF from the stock bottle into a glass bottle to limit repetitive opening of stock bottles. This solution will be used as last dehydration treatment during the clearing procedure. Then, prepare 50, 70 and 80 vol/vol% THF dilutions by mixing and gently shaking pure 100% THF with bidistilled water. 250 ml of each dilution is sufficient to treat one whole body. These prepared solutions can be stored in glass bottles with a solvent resistant lid at room temperature in dark for some weeks. **CAUTION** THF is inflammable, toxic and highly volatile, hence avoid inhalation and contact with eyes/skin by always working in a fume hood, with safety goggles and double layer of nitrile gloves, while handling it. Since THF tends to form peroxides over time, it possesses explosive risk if stored for months or years. Therefore, it should be purchased in max 2 L bottles and it should not be ordered in big quantities to avoid stocking up unused THF in the lab for long periods. To reduce the risk of formation of peroxides, we suggest purchasing THF containing a stabilizer such as 250 ppm BHT (see Reagents section). Both open and unopened/new bottles of THF should be kept at room temperature in a safety cabinet for flammable and explosive substances. Follow the guidelines for peroxide-forming compounds carefully:
(https://www.sigmaaldrich.com/chemistry/solvents/learning-center/peroxide-formation.html).

Delipidation solution

The delipidation solution consists of pure 100% DCM. Since whole-body vDISCO needs at least 250 ml of pure DCM per animal, at the delipidation step the DCM can be poured into the clearing chamber directly from the stock bottle. However, if working with small dissected body pieces, then transfer 200 ml of 100%

DCM from the stock bottle into a glass bottle with a solvent resistant lid to avoid repetitive opening of stock bottles. Use the transferred solution within 1 month. **CAUTION** DCM is toxic and highly volatile. Avoid inhalation and contact with skin/eyes by handling it in a fume hood and by wearing safety goggles and double layer nitrile gloves. Store all DCM solutions in dark and in well-sealed bottles at room temperature in a safety cabinet for solvents.

RI matching solution

We use BABB as RI matching solution. To prepare it, mix 1 volume of 100% pure benzyl alcohol with 2 volumes of 100% pure benzyl benzoate. 300-350 ml of BABB is sufficient for one animal. BABB can be stored for some months at room temperature in the dark in glass bottles sealed with lids resistant to solvents. **CAUTION** Although both benzyl alcohol and benzyl benzoate are reported to have low systemic toxicity and no carcinogenic effects, the first can be very toxic to the eyes, while the second can cause eye irritation. Both are known to be skin irritants. Avoid contact with eyes and skin. Prepare BABB in a fume hood and wear safety goggles and double layer nitrile gloves (see also **Box 1**).

Equipment set up

In whole-body active vDISCO the setting up of the transcardial-circulatory perfusion system is required for the steps of decolorization, decalcification and staining. The perfusion system is constituted as shown in **Figure 4**. To establish it, we used the Ismatec peristaltic pump indicated in the equipment list. This pump can hold 4 pumping channels with one set-up tube per each channel (**Figure 4b,** left); therefore 4 animals can be processed at the same time. For the sake of simplicity, here we describe the procedure to set up one channel with a single reference tube (**Figure 4b**, right and **Figure 4c**, blue arrow). Insert the hose tubing connectors at both ends of the reference tube (**Figure 4c**, red rectangles). Through the tubing connectors, connect the reference tube with 2 additional PVC tubes at each side of the reference tube (**Figure 4c,** magenta arrows), in order to extend the total length of the reference tube. Use parafilm to seal the connecting parts (**Figure 4c,** red boxed insert). Next, cut the tip of the 1 ml Braun syringe and use this tip to connect one end of the tube, which will represent the outflow end during the experiment, with the perfusion needle (**Figure 4c,** yellow insert and **Figure 4d**). When starting the protocol, the needle will inject the vDISCO solutions into the circulatory system of the animal through the left ventricle of the heart. **CRITICAL** Make sure that all the connections are stable. Check and test the integrity of all the components and the absence of leaking points by pumping some water.

Procedure

Sample preparation with PBS and PFA perfusion TIMING 14 h to 1 d

1. Deeply anesthetize the animal by intraperitoneally (i.p.) injecting the MMF triple combination 1 ml per 100g body mass for mouse. Wait some minutes to let the anesthesia set in. Check the pedal reflex of the animal to evaluate the success of the anesthesia. **CAUTION** All the steps involving husbandry, handling and euthanasia (including type of anesthesia) of the animal must follow the appropriate governmental and institutional regulations.
2. If using the Leica Perfusion One system to perfuse the animal, fill one of its pumping tanks with room temperature heparinized PBS (alternatively ice cold) and a second pumping tank with room

temperature 4 (wt/vol)% PFA (alternatively ice cold). Start pumping for 1-2 minutes with the heparinized PBS to fill up the pumping tube and to push out all the air bubbles. **CRITICAL** Air bubbles trapped in the pumping tube should be removed already at this stage, because the pumping of air into the body can block the subsequent circulation of the solutions in the vasculature of the animal, impairing the fixation. Also, the perfusion of the animal can be performed in many other ways: e.g. using an electric peristaltic pump, manually pushing the solutions in the heart with syringes or using gravity perfusion systems. It is up to the operator to choose the perfusion strategy that the operator is most familiar with.

3. At room temperature, intracardially perfuse at pressure ~110 mmHg the anesthetized animal with the heparinized PBS for 5-10 minutes until the blood is flushed out. Then, switch the perfusion with the 4% PFA solution for 10-20 minutes until the animal gets fixed. **CRITICAL STEP** The perfusion step must run flawlessly, otherwise the whole body vDISCO immunolabeling might be impaired. To assess the success of the perfusion in this step, visually check if the liver starts turning yellow (**Supplementary Figure 1a**, cyan dashed line) and the perfusate drains clear from the right atrium. Moreover, the animal should become rigid and stiff starting from the 4th or 5th minute of perfusion with 4% PFA (usually after 25 ml of PFA). **? TROUBLESHOOTING**

4. From this step the procedure will be illustrated differently if the chosen protocol is passive-vDISCO for dissected tissues or organs (option A) or whole-body active vDISCO (option B):

A) Passive-vDISCO staining **TIMING** 2 d to 23 d (excluding the optional decolorization and decalcification treatments, see **Table 2** for timing details)

1. *Sample preparation*

   i. After perfusing with 4% PFA, dissect out the tissue or organ of interest from the animal. **CRITICAL** the content of the gut cannot be sufficiently cleared. The gut can be emptied either by flushing the content through small incisions made in the tissue with a syringe filled with 1X PBS or gently manually squeezed out of the body. **(Supplementary Figure 1b, Supplementary Video 1)**. (**Supplementary Video 2)**.

   ii. Post-fix the dissected organs/tissues in appropriately sized tube in 4% PFA overnight at 4°C. **CRITICAL** The sample must be immersed in at least 5 sample volume of 4% PFA solution., Avoid overfixation to prevent autofluorescence. Label the tubes with solvent resistant ink (permanent pen, graphite pencil) and further cover the label with tape.

From now on, all the following steps will be done with gentle shaking on a shaker or on a rocker: 50-100 mot 1/min with the shaker IKA KS 260 basic, 25 r.p.m. with the rocker IKA 2D digital. These shakers/rockers should be put in the incubator for the steps that require a temperature of 37°C. All the following steps can be done in the same container used for post-fixation.

   iii. After post-fixation, wash the samples with 1x PBS 3 times for 20 minutes to 2 hours for the larger samples (**Table 2**) at room temperature and with gentle shaking.
   PAUSE POINT washed samples can be stored at 4°C in PBS for up 1-2 years. To prevent fungal contaminations add 0.05% sodium azide to the PBS. **CAUTION** Contaminated samples are not usable.

iv. **OPTIONAL** Cut the sample in 500 µm or 1 mm sections using a vibratome and leave them in PBS for immediate use or in PBS + 0.05% sodium azide at 4°C for longer storage (up to 2-3 months).

v. **OPTIONAL** Dissected spinal cords or whole central nervous systems (brain and spinal cord) can be stored straightened using plastic Pasteur pipettes cut in half and fine needles (see **Supplementary Figure 4)**.

2. **OPTIONAL** *Decolorization*

i. For samples containing blood after the PBS/PFA perfusion (dark red color), we provide a decolorization step. To this end incubate the sample gently shaking in decolorization solution (see also **Table 2**). For faster protocol incubation can be performed at 37°C.. Refresh the decolorization solution 2-3 times (e.g. every 3-8 hours) until the sample appears lighter color and the solution remains trnasparent. **CAUTION** This treatment can reduce the overall tissue clearing performance time and decolorization concentration should be optimized to specific samples (see **Table 2**).

ii. Wash thorough at least 5 times with 1x PBS at 37°C to eliminate any traces of decolorization solution. **PAUSE POINT** last washing step in PBS can be extended to overnight.

3. **OPTIONAL** *Decalcification*

i. Decalcify the sample (e.g. bones) by incubation in decalcification solution, at room temperature with gentle shaking until softening of the sample which become flexible. **CRITICAL** The decalcification step can decrease the staining performance

4. Wash 5 times with 1x PBS at 37°C to to eliminate any traces of decalcification solution. **PAUSE POINT** last washing step in PBS can be extended to overnight

5. *Permeabilization*

i. Treat the sample 3 hours to 2 days (adjusted to sample size) with the permeabilization solution at 37°C with gentle shaking (see **Table 2** for timing details).

6. *Staining*

i. Incubate the sample in the staining solution containing the dyes of interest at 37°C for 3 hours-21 days with gentle shaking and protected from light. The concentration of the dyes, the final volume and the timing used for the incubation must be adjusted to the expression of the target and to the size of sample as shown in **Table 1-2**. **CRITICAL STEP** If using nanobodies that have stability issues as indicated in **Table 1**,we recommend using the **mild-vDISCO protocol** at this step wash the permeabilized samples with the washing solution 30 minutes 3 times then incubate in the **mild-staining solution added with the dyes** (**Table 3**).
Sample containers must be tightly sealed (e.g. cover the lid container with parafilm) to prevent evaporation.
CRITICAL Validate the efficiency of the nanobody in sections before using in vDISCO.

ii. Wash the sample with the washing solution 3 times for 10 min – 1 h (adjusted to sample size) at room temperature, protected from light and with gentle shaking. **PAUSE POINT** last washing step in PBS can be extended to overnight.

iii. Wash the samples with 1x PBS 3 times for 10 min – 1 h (adjusted to sample size) at room temperature, protected from light and with gentle shaking.

PAUSE POINT Stained samples can be stored in PBS for up to 1 day at 4°C protected from light.

B) Whole-body active vDISCO staining TIMING 12-16 d

A summary of the steps is shown in **Table 4**

1. *Sample preparation*

   i. After the PBS and 4% PFA perfusion of the animal, the skin and the eyes are removed (**Supplementary Figure 1c**). Moreover, in order to achieve better post fixation of the brain, a piece of the occipital bone (back of the skull) is also removed (**Supplementary Figure 1d**, green dashed region) and the hard palate (**Supplementary Figure 1e**, cyan dashed region) of the animal was opened (being careful of not damaging the tissue above). **CRITICAL** The gut content (food and feces) cannot be cleared, thus later impairing the imaging. If necessary (e.g. in whole body studies involving the gut) the content can be removed by flushing it out with 1x PBS using a syringe through 3-4 small gut incisions **(Supplementary Figure 1b, Supplementary Video 1)**. If the feces are hard, especially in the last tract of the large intestine, they can be gently squeezed out from one of the cuts, delicately pushing the intestine outer wall with the tips of the fingers (**Supplementary Video 2**). If the clearing of the whole mouse with intact skin is desired and the animal has fur (e.g. albino or mice with BL6 background), shave off the hair with a razor blade, being careful of not damaging the skin. Alternatively, use a commercial hair removal cream according to manufacturer's protocol (**Supplementary Figure 1f-h**).

   ii. Wash the whole body extensively with PBS to clean the body from hair and digested food as much as possible, otherwise hair and food residues can clog the pumping system used later in the staining procedure.

   iii. Post-fix the specimen in 4% PFA at 4°C for 1 day in 50 ml tubes or bigger plastic containers. **CRITICAL** The sample must be completely immersed in the 4% PFA solution. Avoid over-fixation because it can increase the tissue autofluorescence.

   iv. After post-fixation, wash the samples with 1x PBS 3 times for 1 hour each at room temperature and with gentle shaking. **PAUSE POINT** washed bodies can be stored at 4°C in PBS for up to 4 weeks, in PBS+0.05% Sodium Azide for up to 1-2 years. **CAUTION** Do not use samples where bacteria or fungal contamination is observed.

2. *Sample+perfusion system set up*

   i. Place the body of the animal in the 300 ml glass chamber (**Figure 4e**) and put it close to the peristaltic pump (**Figure 4f**).

   ii. Place the sucking end of the pumping tube inside the glass chamber until the tip touches the bottom of the chamber (**Figure 4e**, bottom, black arrow).

   iii. Fill the chamber with 1x PBS with an amount that can cover the body.

   iv. Start the pumping for 2-3 minutes to make sure that the entire tube is filled with PBS with no air bubbles.

   v. Using the sample/tube holders from **Figure 4a**, fix the whole pumping tube with an angle that has the perfusion needle directed into the glass chamber (**Figure 4f**).

   vi. Insert the perfusion needle (which was placed onto the outflow end of the tube) inside the same entry point hole that was created during the PBS-PFA perfusion step at the level of the left ventricle (**Figure 4g,h**). **CRITICAL STEP** Be extremely gentle at pushing the needle inside the hole. Don´t force the needle because, although the perfusion needle ends with a round tip

and not a sharp tip, it can still damage the tissue around the hole. Don´t push the needle too hard or too deep because it can break the organ. The hole must be just big enough for the perfusion needle to get in. If the hole becomes too big, the perfusion solution can immediately leak out from the hole. This will result that the solution will not circulate in the vasculature. **? TROUBLESHOOTING**

vii. **OPTIONAL** Remove some PBS from the chamber to expose only the heart to the air. While doing this, make sure that the sucking end (inflow end) of the tube is constantly immersed in PBS. Add 1-2 drops of the liquid Pattex or Toolcraft superglue onto the point where the needle goes into the heart in order to fix the needle in place, to seal the hole and keep the perfusion pressure constant (**Figure 4i**, left). Let the glue dry for some minutes (**Figure 4i**, right). When the glue has dried, cover the animal back with 1x PBS. **CRITICAL** This step is indicated as optional at this point, because later the perfusion with the decolorization solution might detach the glue from the heart. However, the solidified glue will still hold onto the needle and can act as a cover on the hole to keep the pressure. If it is observed that the pressure doesn´t seem to be kept well while pumping (e.g. a lot of flow coming out directly from the hole), then the adding of the glue can be done not at this stage but just before the staining step (described later).

3. *Decolorization*

i. Set the peristaltic pump with a pumping rate of 45-60 r.p.m which is 160-230 mmHg. Start the pumping and perfuse the animal with the 1x PBS contained in the chamber for 2-3 hours 2 times (one time can be left overnight) at room temperature. This procedure washes the body before pumping other solutions. The perfusion will work as follows: the needle (outflow) injects the PBS into the body, while the other ending of the perfusion tube (inflow) collects the solution exiting from the mouse body, pumping it back into the animal in a closed recirculation loop. **CAUTION** Always protect skin and eyes when handling the pumping system. Sudden sprays of solution during pumping, in particular if the pressure is too high, can reachthe operator's eyes. The indicated pressure is higher than the physiological one, in order to better push the reagents into the tissue. However, very carefully adjust the pressure based on the feedback from the body of the animal (smaller animals require less pressure). When the pressure is too high, the solution might get spurted out from the openings of the animal, or the heart, lungs, blood vessels might burst. In these circumstances stop the pumping immediately. Before turning on the pump again, you can reduce the pumping pressure or make a bigger cut on the right ventricle (**Figure 4j**). A pumping performed with a pressure that is too high can damage the vasculature, by creating leaking breaks and thus jeopardizing the whole experiment. **CRITICAL** From this point, everyday refresh "the pumping reference tube slot": the reference tube has 2 slots for pumping, the pumping can work alternatively on one of the 2 slots (**Figure 4k,** top). After many hours of pumping with high pressure, the running slot can get deformed by the movement of the gearwheel of the pump, thus reducing the performance of pumping. Thereby, we highly recommend alternating the slots every 12 hours (the operator can do it once in morning and once in the evening before leaving the lab) (**Figure 4k**, middle and bottom and **Supplementary Video 3**).

ii. After washing with PBS, the perfusion should continue with the next solution. The exchange of the perfusion solution is performed without touching the perfusion set up or the animal:

first, stop the pumping, then completely suck out the PBS with a 50 ml serological pipette inserted into a pipette boy (**Figure 4l**). **CRITICAL** This operation must be done by avoiding the formation of bubbles inside the perfusion tube. If the sucking end of the tube is well pushed into the chamber with the tip touching the bottom of the chamber, when removing the whole PBS from the chamber, some PBS (about 1 mm in depth) will anyway remain inside the chamber. The surface tension of this remaining PBS at the level of the end of the sucking tube will prevent the formation of air bubbles inside the tube (**Figure 4e,** bottom). Therefore, be careful to not disrupt this surface tension by e.g. accidentally moving the sucking tube.

iii. Pour the decolorization solution inside the glass chamber, by covering the animal. Start the pumping with the same pressure optimized in the previous step and perfuse for 2 days at room temperature. In these 2 days exchange the decolorization solution whenever it turns into a strong yellow color (about every 12 hours, **Figure 5a**), indicating that the heme is being successfully eluted out from the body. You will see that on the last exchange, the solution will stay colorless or turn into pale yellow; in both cases the sample is ready for the next step. **CRITICAL STEP** The perfusion of the decolorization solution is a good indicator of the performance of the whole body perfusion system: after 2 days of decolorization the spleen becomes pale beige color (**Figure 5b**) and the body whiter**. ? TROUBLESHOOTING**

4. *Decalcification*

i. With the same procedure explained in the previous section, after decolorization, exchange the decolorization solution with 1x PBS and perfuse 3 times for 2-3 hours at room temperature to wash the decolorization solution out from the body. **PAUSE POINT** If necessary, it is possible to keep the body under 1x PBS perfusion for max 2 days (e.g. over the weekend). In this pause point, the daily exchange of the reference tube slot is not required, yet, it must be performed as soon as you want to proceed with the next step.

ii. After washing, exchange the PBS with the decalcification solution in the same way as described before and perfuse with the decalcification solution for 2 days at room temperature. The refreshing of the decalcification solution is not required. To assess the success of the decalcification process, check if the body of the animal and its skeleton bend easily: test by bending one of the limbs with a pair of tweezers. **? TROUBLESHOOTING**

iii. After decalcification, wash again by perfusing with 1x PBS 3 times for 2-3 hours at room temperature. **PAUSE POINT** If necessary, it is possible to keep the body under 1x PBS perfusion for max 2 days (e.g. over the weekend). In this pause point, the daily exchange of the reference tube slot is not required, yet, it must be performed as soon as you want to proceed with the next step.

5. *Permeabilization and staining*

i. Replace the PBS with the permeabilization solution and perfuse with the permeabilization solution for half day at room temperature.

ii. After permeabilization, turn off the pump, take a 0.22 µm filter and wet it with some permeabilization solution using a syringe (the wetting will reduce the formation of bubbles inside the pumping tube, **Figure 5c**). Then take the sucking (inflow) end of the perfusion tube and connect it to the filter (**Figure 5d**). Finally place the tube+filter inside the chamber (**Figure 5e**). **CRITICAL** The filter has the function to filter the staining solution before entering the body of the animal, preventing in this way the accumulation of dye aggregates into the sample.

Periodically check if the filter still functions, because sometimes after a couple of days the solution cannot be pumped anymore because the filter is blocked. Simply replace the filter with a new one. **? TROUBLESHOOTING**

iii. With the pump still turned off, suck out the permeabilization solution from the chamber using the serological pipette as shown before in **Figure 4l**. Pour fresh permeabilization solution into the chamber. From now on, this fresh permeabilization solution will be called staining solution since it will contain the dyes. **CRITICAL STEP** At this point if you have already put the liquid superglue as indicated in the optional point of the decolorization section (option B, point 2. vii.) and the glues still holds well, then proceed with covering the whole animal with 250 ml of staining solution, otherwise the level of the fresh staining solution should not reach the heart because it is very important to apply the liquid superglue at this stage, by following the procedure described in the optional point of the decolorization section described before (option B, point 2. vii.): briefly add 1-2 drops of superglue onto the point where the needle goes into the heart, let the glue dry and cover the animal completely with 250 ml of staining solution, and make an incision in the right ventricle (**Figure 4i,j**).

iv. Add the dyes into the staining solution (**Figure 5f**) using a pipette. See **Table 1** for the tested nanobodies used in whole-body vDISCO and their concentrations. If needed, perform counterstain of nuclei using a nucleus dye such as PI: add 290 µl of PI from the stock bottle into the staining solution. **CRITICAL** The nanobody has to be previously validated in sections.

v. Cover the chamber with aluminum foil (it doesn´t necessarily need to be tight around the chamber), to minimize light exposure (**Figure 5g**).

vi. Turn on the pump and perfuse the animal for at least 6 days with the staining solution containing the dyes. At the same time place the infrared lamp 20-30 cm from the chamber and direct the infrared light to it (**Figure 5g**). The infrared light will heat up the solution to about 28-30°C, increasing in this way the molecular movement of the dyes in order to achieve a better staining. Any other way to increase the temperature can be used, for example by putting the pump and the whole setting in a temperature controlled warm room or onto a hot plate. If the PI is added, different organs of the body such as intestine, lymph nodes and thymus will turn pink (**Figure 5h**). **CAUTION** The infrared lamp is not designed for continuous use over many hours. Instead, it can overheat after prolonged usage. After 10-12 hours of usage we recommend switching the lamp off until it cools down, before switching it on again. **CRITICAL** Check every day the level of the solution in the chamber: if the level decreases due to evaporation, fill it back with distilled water only. **? TROUBLESHOOTING**

vii. After 6 days remove the perfusion tube+needle from the heart, by delicately detaching the glue. Take the body out from the chamber and place the body in a 50 ml tube or in a bigger plastic container with lid. Fill the tube or the container with fresh staining solution plus an additional 5µl of only nanobody (**Figure 5i**).

viii. Passively incubate the tube at 37°C or at room temperature for 1-2 days with gentle shaking and protected from light for additional staining and propagation of the dyes into the tissue. The choice of the temperature for this step is based on the stability of the nanobody (**Table 1**), for example we recommend room temperature incubation for nanobodies that would normally require passive mild-vDISCO for the staining of dissected organs.

ix. Meanwhile, discard the filters and the solution left in the glass chamber. Wash the container with distilled water, then fill the chamber with 1x PBS and put it back to the pumping set up. Turn on the pump (with no sample inside the chamber) for 30 minutes, exchanging the PBS 2-3 times to wash the tubes and remove residues of dyes.

x. After 1-2 days, place the animal back into the glass chamber of the (now) clean perfusion system and wash the body by perfusing it with the washing solution 2 times for 2-3 hours at room temperature. From this step the filter is not needed anymore.

xi. In the end, wash the body by perfusing it with 1x PBS 2 times for 3 hours at room temperature. **PAUSE POINT** Stained samples can be stored in PBS for up to 1 day at 4°C in dark. **? TROUBLESHOOTING**

Clearing **TIMING** 1-4 d

As mentioned before, the clearing procedure is performed exactly in the same way for both whole bodies and dissected samples: through passive incubations with organic solvents.

1. After staining, the samples are immersed in 1x PBS in containers that are resistant to organic solvents (tubes or glass chambers) and ready to be cleared. In case of small tubes (e.g. 5ml tubes), suck out the PBS with a Pasteur pipette and immediately add inside 50% THF, which is the first solution of the dehydrating series. In case of bigger containers (e.g. 50 ml tubes or glass chambers), directly pour out the PBS into a big beaker for waste, by being careful of not accidentally dropping the sample in it; then, immediately pour the 50% THF into the container carrying the sample, being careful of not spilling around the solutions. The amount of the clearing solution must almost fill the plastic tube (e.g. 4-4.5 ml into 5ml tubes) or cover the whole body in the glass chamber (about 200-250 ml). If clearing whole bodies, the intestine should be pushed into the abdomen to reduce the thickness of the cleared sample (**Figure 5j**). This will later facilitate the imaging. It is possible to put onto the body a glass lid of small staining jars as a weight with the same purpose of reducing the thickness of the body (**Figure 5k**, magenta arrowheads). **CAUTION** All clearing solutions possess different grades of toxicity and irritation capability; therefore always perform all the steps of the clearing protocol in a fume hood, wearing safety goggles and double layer of nitrile gloves.
2. After closing the containers very well or having put the lid on the glass chamber, incubate the sample in 50% THF for 20min-12h (see **Table 2,4** for timings based on the sample size) with gentle shaking, at room temperature and in dark (for example by covering the containers with aluminum foil) (**Figure 5k**).
3. After incubating in the first dehydration solution, replace the 50% THF with 70% THF with the strategy described above and incubate again for 20min-12h (**Table 2,4**) with gentle shaking, at room temperature and in dark. **CAUTION** Discard all the solvents following the institute regulations.
4. Follow the same procedure for the incubations in 80% THF, 100% THF and again 100% THF. Optionally seal the lid of the glass chamber with parafilm for the incubations with pure THF to prevent evaporation of the solvent (**Figure 5k**, right).
5. With the same procedure, exchange 100% THF with 100% DCM and incubate the sample in DCM for 10min-3h with gentle shaking, at room temperature and in dark. Optionally seal the lid of the glass chamber with parafilm for the incubations with pure DCM to prevent evaporation of the solvent. **CRITICAL** Use a set of Pasteur pipettes dedicated to each kind of solvent and its dilutions

(e.g. one pipette for THF dilutions, one for THF waste, one for DCM etc.) to prevent contaminations.

6. In the end, follow the same strategy to replace DCM with BABB: discard the DCM and add some BABB into the container, then manually shake the tubes or the glass chamber for 4-5 seconds to wash away the remaining DCM left in the tube, then immediately discard this BABB solution.

7. Add fresh BABB to the container by completely covering the sample and incubate for 20min-12h with gentle shaking, at room temperature and in dark, until the sample becomes visually transparent (**Figure 5l**). The change of color into a brown shade will not affect the imaging. **PAUSE POINT** Cleared samples can be stored for months or years in BABB at room temperature in dark without significantly losing fluorescence. Unimaged whole bodies can be kept for some weeks in the glass chambers used for clearing, however for long-term storage they should be moved in plastic containers filled with BABB and with anti-leakage lids (e.g. 50 ml tubes). Small samples can be stored in the same tubes used for clearing. **CRITICAL** Label the tubes with permanent pens, cover the text with transparent sticky tape to protect the label. **? TROUBLESHOOTING**

Imaging

1. *2D epifluorescence microscope (AxioZoom) imaging* **TIMING** 5-15min

   i. For this kind of imaging, the sample should be kept in a container that holds enough BABB to keep the sample completely immersed. For example small samples can be placed in smaller transparent solvent resistant containers with a flat bottom (e.g. glass petri dishes) (**Supplementary Figure 5a-c**), while the whole cleared body can be placed in the same glass chamber used for clearing (**Supplementary Figure 5d**). **CAUTION** Perform all the next imaging steps wearing nitrile gloves and safety goggles.

   ii. Put the glass container with the sample and without the lid under the epifluorescence microscope coupled with the 1x objective (**Supplementary Figure 5b-d**).

   iii. Place the sample in the chamber as straight as possible and be careful of avoiding accidental movements of the sample. **? TROUBLESHOOTING**

   iv. Focus on a part of the sample where it is known there is signal. **? TROUBLESHOOTING**

   v. Adjust the zoom based on the details that you want to see. For example select zoom factor x7 for the 2D whole body reconstruction. Normally small cells such as microglia can be imaged with zoom about x63 or higher.

   vi. Start scanning the sample by taking individual 2D images over the sample and by covering the entire sample. In this operation, move the chamber in x-y directions very slowly to prevent any accidental sample movement. **CRITICAL STEP** Make sure that consecutive 2D images have overlapping regions at the edges of the images to facilitate the stitching.

   vii. Save each scan as multi-channel scans if doing multi-color imaging.

2. *Light-sheet LaVision Biotec-Miltenyi Ultrmicroscope II imaging* **TIMING** 2-12h

   Use this light-sheet for high resolution imaging of organs, dissected body parts or small organisms (e.g. embryos)

   i. Mount the desired zoom body unit onto the microscope by following the supplier instructions. **CAUTION** Perform all the next imaging steps wearing nitrile gloves and safety goggles.

   ii. Pour BABB in the imaging chamber, filling half of the chamber.

iii. Mount the sample onto the sample holder: small samples such as brains can be simply mounted using the screwing system provided by the microscope supplier (**Supplementary Figure 6a**) or plunged onto Micro-Fine Ultra needles attached to the sample holder[16] (**Supplementary Figure 6b-e**), bigger samples such as chest or the abdomen can be mounted on the sample holder using the superglue (**Supplementary Figure 6f-j**). In case of using the latter method: first attach a piece of black sticky tape onto the surface of the sample holder (**Supplementary Figure 6g**), then apply 1 drop of Maxi-Cure super glue onto the tape (**Supplementary Figure 6h)**, then inject 30-40 µl of the Insta-Set Accelerator over the super glue using a 1ml syringe + needle (**Supplementary Figure 6i)**, immediately place the sample onto the glue and hold it for 1 minute until it is stabilized (**Supplementary Figure 6j**). In this way, the removal of the glue after the scan can be easily done by detaching the tape, without leaving any glue residue on the sample holder. **CRITICAL** This operation should be carried out as fast as possible because when the tissue is not soaked in BABB, air can go into it, thus creating air bubbles. Be gentle while grabbing the samples with tweezers and don´t squeeze them in order to prevent the accumulation of air bubbles inside. If necessary, the samples can be trimmed and cut with scissors or the motorized dental blade, the cutting should be performed immersing the sample and the scissors (not the motorized blade) in BABB to prevent the entrance of air bubbles. If using the super glue, the surface of the sample that will be in contact with the glue should be cleaned a bit from BABB by wiping it 2-3 times on a piece of paper tissue (e.g. Kleenex) before mounting it. We recommend this glue, because it doesn't leave residues or affect the tissue after imaging. For the removal of bubbles see **Supplementary Figure 6p-r** **? TROUBLESHOOTING**

iv. Place the sample holder with the sample inside the imaging chamber and align it to the chamber.

v. Pour additional BABB into the imaging chamber to cover the sample. **CAUTION** Do not fill the chamber completely to avoid overflowing of BABB, because the spillage of BABB into the mechanical components can severely damage the microscope.

vi. Select the correct filter set for your fluorophore in the software.

vii. Turn on the excitation light and move the sample in z direction until the upper surface of the sample is illuminated by the light-sheet.

viii. Mount the objective of interest. We normally use the 4x immersion objective for the scan of organs that can fit in a tiling scan of maximum 3x4 (e.g. whole brain or smaller samples), the 2x immersion objective for other body parts (limbs, chest etc), the 12 x and the 20x immersion objectives to detect very small details such as single cancer cells, dendritic spines or the structure of microglia ramifications.

ix. By using the lowest zoom provided by the zoom body, slowly lower the objective towards the sample until the structures of the sample appear on the computer screen. **CAUTION** If using immersion objectives, make sure that while lowering the objective into BABB there is no overflow of it, otherwise use a Pasteur pipette to suck out some BABB from the imaging chamber. Water immersion objectives such as Zeiss 20x can be compatible with BABB but we strongly recommend to talk to the supplier or test the objective with BABB before using them in a regular scan. **? TROUBLESHOOTING**

x. Move the objective slightly up and down to adjust the focus. **? TROUBLESHOOTING**

xi. Adjust the microscope setting based on the sample. For example for processed *Thy1*-GFPM brain we used a z-step of 4µm, double-sided light-sheet, NA 0.025, light-sheet width 60%, exposure time 100.00 ms, tile scan 3x3 with 13% of overlap, 5000 µm of z-range. **CRITICAL** The laser power and the exposure time should be optimized by never reaching the saturation of the camera. **? TROUBLESHOOTING**

xii. Adjust the alignment of the 2 sides of the light-sheet. For small or thin samples such as a spinal cord, one –sided light-sheet is sufficient to cover the entire sample.

xiii. Adjust the laser power and the chromatic correction focus of the autofocus box for the other channels, if performing multi-channel imaging.

xiv. Start the scan into the computer hard drive by using 2 sided-light sheet or 1 sided-light sheet based on the size of your sample. Try to name the scan in a consistent way for example indicating the number of the animal or the date of preparation, animal line, body part, staining protocol and dye, excitation wavelength (to indicate the filter set used), objective magnification, zoom, tiles, overlap, z-step (e.g. GFPM248-DP2892019-brain-vDISCO-647nGFP-PI-545640ex-4x-3x3-13o-4umz). For signal intensity quantification it is recommended to take note of the laser settings used per each channel and the exposure time.

xv. Take a screenshot of the Imspector software with the 'info' icon switched on to record the settings after checking all the parameters. Then, start the scan without touching the light-sheet microscope or running other software in the hosting computer to avoid potential interruptions. Wait until the scanning and imaging recording is completed.

3. *Light-sheet imaging with the LaVision Biotec-Miltenyi Blaze microscope* **TIMING** 6-7d

Use this light-sheet microscope coupled with the 1.1x immersion objective for high resolution imaging of large samples such as pig pancreas[48] or intact mouse bodies.

i. Pour BABB into the imaging chamber, by filling half of the chamber. **CAUTION** Perform all the next imaging steps wearing nitrile gloves and safety goggles.

ii. Mount the sample onto the sample holder with the Maxi-Cure superglue + accelerator as shown in **Supplementary Figure 6k-o**. In case of a whole body, you can either start mounting the body in prone position facing down or in supine position facing up. First, attach two pieces of black sticky tape onto the surface of the sample holder (**Supplementary Figure 6k,l**). Suck 50-70 µl of the Insta-Set Accelerator solution with a 1 ml syringe and keep it for later. Next, apply 3-5 drops of Maxi-Cure super glue onto both pieces of the tape (**Supplementary Figure 6m**). After that, inject about 20-30 µl of the Insta-Set Accelerator into each drop of the glue (**Supplementary Figure 6n**), in a fast manner. Place the body onto the sample holder and hold it for few minutes until the glue cures **(Supplementary Figure 6o)**. The removal of the glue after the scan will be easily done by detaching the tape. **CRITICAL** Avoid leaving the sample outside of BABB for too long and perform this operation fast. When the sample is left outside of BABB, air can go into the sample and create air bubbles within the sample. Be gentle while grabbing the samples with tweezers and don´t squeeze them to avoid the accumulation of air bubbles inside the sample. For the removal of air bubbles see **Supplementary Figure 6p-r** **? TROUBLESHOOTING**

iii. Place the sample holder with the sample inside the imaging chamber.

iv. Pour additional BABB into the imaging chamber to cover the sample. **CAUTION** Do not completely fill the chamber to avoid overflowing of BABB, because the spillage of BABB into the mechanical components can severely damage the microscope.

v. Select the correct filter set for your fluorophore in the software.

vi. Turn on the excitation light and move the sample in z direction until the upper surface of the sample is illuminated by the light-sheet.

vii. Using the 1x zoom provided by the zoom body, slowly lower the objective towards the sample until the structures of the sample appear on the computer screen. **CAUTION** Make sure that, while lowering the objective into BABB, there is no overflow of it, otherwise use a Pasteur pipette to suck out some BABB from the imaging chamber. **? TROUBLESHOOTING**

viii. Move the objective slightly up and down to adjust the focus. **? TROUBLESHOOTING**

ix. Adjust the microscope settings. For example, to cover a whole adult mouse, set 3x8 tiles of scans with 35% of overlap, laser power 10-15%, z-step 6µm, light-sheet NA 0.035, light-sheet width 80%-100%, exposure time 80.0-120.0 ms, 11 mm of z-range. Settings can be adjusted based on the sample. **CRITICAL** The laser power and the exposure time should be optimized by never reaching the saturation of the camera, while the number of tiles should be set based on the sample size. **? TROUBLESHOOTING**

x. Start the scan by saving into the computer hard drive and by using 2-sided light-sheet. Try to name the scan in a consistent way for example indicating the number of the animal or the date of preparation, animal line, staining protocol and dye, excitation wavelength (to indicate the filter set used), objective magnification, zoom, tiles, overlap, z-step (e.g Prox1-2-DP310919-ventral-vDISCO-647nGFP-PI-545640x-1p1x-3x8-25o-6umz). For signal intensity quantification it is recommended to take note of the laser settings used per each channel and the exposure time.

xi. Adjust the laser power and the chromatic correction focus of the autofocus box for the other channels, if performing multi-channel imaging.

xii. After imaging one half side (ventral or dorsal) of the entire body (or sample), remove the body from the sample holder, discard the black tape and clean the sample holder surface by wiping with a Kleenex and 80% Ethanol.

xiii. Flip the body or sample and mount it onto the sample holder with the super glue + accelerator method indicated before. **CRITICAL** When in prone position the animal might result tilted in z because the thickness of the head of the animal including the snout is higher than the thickness of the belly. This issue can be solved by creating a sort of platform to lift the belly part: apply onto the black tape at least 3 layers of super glue plus accelerator in the location where the belly will be put. In this way the whole dorsal side will be parallel to the sample holder surface in z.

xiv. Scan now the other side in the same way as described before.

4. *Light-sheet imaging with a modified mesoSPIM* **TIMING** 10h to 2d

Standard mesoSPIM setups have a maximum travel range of 52 × 52 × 102 mm which can be limiting for imaging large samples such as entire cleared mice. We therefore modified an existing mesoSPIM with larger sample stages to increase the travel range to 100 × 200 × 100 mm to allow tiled acquisitions without remounting the sample **(Supplementary Figure 7a-b)**. To reduce the number of required tiles while retaining µm-level sampling, we also integrated a sCMOS camera

with 3.75× more pixels compared to the published mesoSPIM version (15 MP vs. 4 MP). The modified mesoSPIM allows us to perform two channel acquisitions of whole mice with 4.7 × 4.7 x 10 µm sampling within 9 hours.

To simplify sample handling, we mount large samples in custom large cuvettes - e.g. 40 × 40 × 120 mm for a vDISCO-processed adult mouse **(Supplementary Figure 7c-e).** Mounting the sample in a large cuvette provides optical access from all four cuvette faces which simplifies multiview acquisitions.

i. Switch on the mesoSPIM and start the mesoSPIM-control software

ii. Lower the sample into the cuvette and fill it with BABB solution.

iii. To stabilize the sample inside the cuvette, insert an appropriately sized (e.g. 40.5 mm for a 40 mm cuvette) crossbar between the cuvette walls above the sample and gently press it down onto the sample. The crossbar should be made from BABB-resistant material, e.g. 3D-printed from nylon (PA-2200) **(Supplementary Figure 7d)**

iv. Attach the lid of the sample cuvette **(Supplementary Figure 7c)**.

v. Insert the sample into the microscope by attaching the lid magnet to the rotation stage **(Supplementary Figure 7e)**. **CAUTION** Be careful when moving the sample in XYZ to avoid crashing into microscope components. The magnetic sample holders usually provide a safety zone – when touching microscope components such as the scan lens mounts, the cuvette will first gently tilt before cracking. By slowly reversing the movement, it is thus possible to return the sample cuvette to a safe location.

vi. Adjust the position of the sample by translating the sample via the mesoSPIM-control software until the fluorescent image can be acquired by the camera. **CRITICAL** Be aware that when translating the sample in Z, the detection focus needs to be changed as well to keep the light-sheet in focus.

vii. **CRITICAL** Move the sample around to ensure that the sample and the sample holder will not collide the imaging chamber in the desired scanning/tiling range.

viii. Before starting acquisition, the rotation of the cuvette needs to be aligned to minimize the offset of the illumination from the left and the right side. If the cuvette walls are not perpendicular to the light-sheet, refraction will lead to the left and right light-sheets illuminating different parts of the sample. To make the cuvette wall perpendicular to the light-sheet propagation direction, use a white piece of paper (e.g. a business card) to check where the back-reflection from the cuvette wall enters the scan lens. Then, rotate the cuvette using the rotation controls in mesoSPIM-control to superimpose the back-reflection with the excitation beam. If different sample rotation angles are required for the left and right patch, co-align the light-sheets according to the mesoSPIM wiki (https://github.com/mesoSPIM/mesoSPIM-hardware-documentation/wiki/mesoSPIM_coalignment)

ix. Run the tiling wizard in the mesoSPIM-control software, following the instructions to set up the range of image scanning, filters, lasers and to specify the filenames and path for saving data. For the 0.9× objective, typical X and Y offsets between adjacent tiles are 12000 µm and 20000 µm, respectively. For each channel, you need to set a start and end focus. When acquiring a z-stack, mesoSPIM-control will linearly interpolate the focus position between the start and end points.

x. **CRITICAL** For whole-body stacks, it is often not possible to properly focus the detection path at the start and end points as both are commonly located outside of the sample. Therefore, mesoSPIM-control provides a focus-tracking wizard that allows to extrapolate the correct focus trajectory from two points inside the sample. After setting up the tiling pattern, preview a tile using the corresponding button in the acquisition manager window. Then, run the focus tracking wizard and move to a first Z-position inside the sample, manually focus the detection path in live mode and mark the position. Repeat the same procedure at a second Z-position. The wizard then allows you to apply the calculated focus trajectory to a selected subset of stacks. (For example only to stacks using a specific excitation wavelength).

xi. The preview button in the acquisition manager can be used to check whether individual tiles in the acquisition manager are set up properly. **CRITICAL** For example, it is advisable to check if the laser intensity is set up correctly to avoid saturated regions of the sample. Ideally, the brightest sample regions are known from previous acquisitions. In addition, the tunable lens parameters (ETL parameter tab in the main window) should be checked to ensure that the light-sheet is configured to be as thin as possible.

xii. Toggle the "Run acquisition list" button to start the scanning. mesoSPIM-control will show two progress bars: The top one shows the progress of the currently running stack and the bottom one the progress of the whole tiling scan. The predicted time estimate for the whole tiling scan will be continuously updated.

xiii. If desired, the sample can then be rotated by 90° or 180° to perform a multiview acquisition.

5. *Lightsheet Z.1 microscope imaging of a mouse brain* **TIMING** 10-30 min

Imaging with Lightsheet Z.1 is advisable for fast acquisition of high-quality images of single mouse organs.

a. Switch on the microscope, start the ZEN software and mount the objectives required for the image acquisition
b. Glue the cleared brain to the Lightsheet Z.1 sample holder (Suppl. Fig. 8a) and mount the sample holder into the microscope. Drive the sample holder to the upper-most position to avoid a collision of the chamber with the sample
c. Fill the chamber with the clearing solution (e.g. BABB) and insert it into the microscope
d. Lower the sample and position it in front of the detection objective, rotate the sample to the desired angle (Suppl. Fig. 8b). It is most convenient when the horizontal plane of the brain is in the XY plane of the microscope (directions are indicated in the ZEN software). Monitor the movement of the sample with the integrated door camera.
e. Find the focus in the sample using the Near-IR pseudo-bright-field mode and readjust the rotational position, if there is need for refinement (Suppl. Fig. 8c)
f. Set up Tracks for imaging by adjusting the laser intensity, exposure time, zoom and activate the Pivot scanner to maximally reduce the stripe artifacts in the images. Use simultaneous two channel imaging to reduce the total time of the experiment.
g. Define the Z stack and the Tile scan for the entire brain
h. Start the image acquisition.
i. Save the data for further processing.

6. *Inverted confocal microscope imaging* **TIMING** 1-3h

Use this microscope in case of needing higher resolutions, for example to image in more detail particular regions dissected from the whole body, or when lacking a light-sheet system.

i. Cut your sample and place it onto the glass slide of a glass bottom dish (**Supplementary Figure 5e,f**).

ii. Cover you sample with few drops of BABB (**Supplementary Figure 5e,f**). It is not necessary that the sample is completely submerged in BABB, as long as the interface between the sample and the glass has BABB. It is not necessary to close the petri dish or to seal it, because BABB has a high evaporation point and doesn't dry out for several days.

iii. Mount the glass bottom dish onto the sample holder of the microscope (**Supplementary Figure 5g**). **CAUTION** MatTek glass bottom dishes are relatively resistant to BABB, even when BABB touches the plastic of the dish or the glue that seals the plastic part with the glass dish. MatTek glass bottom dishes are able to stand the BABB for more than 4-5 hours of imaging without leaking. However BABB will eventually melt the dish after some time. We recommend testing the resistance of the glass bottom dishes with BABB before mounting them onto the microscope. Be careful of an accidental leakage of BABB onto the microscope because BABB can severely damage the objectives and the mechanical parts of the microscope.

iv. Select the desired objective. A long working distance objective such as the 25x Leica would be ideal to cover large depths in transparent samples.

v. Adjust the settings of the scan: channel, gain and laser power, speed, averaging, bits, frame size, tiles etc.

vi. Activate the online stitching.

vii. Start the scan.

viii. Save the scan in CZI format which will store all the metadata available.

Image processing and visualization

1. *Processing of 2D epifluorescence microscope (AxioZoom) scans* **TIMING** 30min-1h

i. From each scan export the single 2D image as "merged channels image" which is RGB. In this way the information of the different imaging channels (if doing multi-color imaging) will be saved together. For example GFP channel (background signal) in green, RFP channel in red, and far-red channel in blue. The signal from each separate channel can be extracted as single color later in Fiji using "Split Channel" function.

ii. In Adobe Photoshop CS6 software go to "File" → "Automate" → "Photomerge".

iii. Select "Reposition" and import all the files (previously exported images) representing your tiles.

iv. Stitch the final image by repositioning the tiles and by changing the opacity of them whenever merging the overlapping regions.

v. Group the stitched tiles in a single Photoshop layer.

vi. Save the stitched file in Photoshop format as .PDD, *.PSD and you can export the stitched image as .jpeg or .tiff file and load it in Fiji for visualization, analysis and the separation of the individual channels.

2. *Processing of Light-sheet microscope (LaVision Biotec-Miltenyi) scans* **TIMING** 2h-6d

i. The LaVision Biotec-Miltenyi light-sheet microscopes save the scans as series of TIFF files in separate/not stitched sequences in one folder.

ii. Stitch the different tiles from one scan/folder with Fiji using the function located in "Plugins" → "Stitching" → "deprecated" → "Stitch Sequence of Grids of Images" (**Supplementary Figure 8a**).

iii. Fill the "Stitch Image Grid Sequence" with the information about grid size, overlap, input directory, output directory and start positions (**Supplementary Figure 8b**).

iv. In "file names": copy the name of one of the images from the scan and replace [00 x 00] with [{yy} x {xx}] and Z0000 with Z{zzzz}.

v. To have a first preliminary check of the result of the stitching, select the option "create only preview", and set "grid size z" = 1, "start x" = 0, "start y" = 0, the "start z" = a number of an optical slice deep in tissue that contains data from each tile. This will yield the correct parameters for all tiles.

vi. If the stitched result looks fine, generate the first stitched image by unclicking "create only preview" and start stitching. **CRITICAL** If there are mistakes in x-y dimensions, manually correct the positioning of the tiles in 2D, by using the TrakEM2 plugin of Fiji. **OPTIONAL** stitching for all sections can be performed with this plug-in by simply changing the "grid size z" to the total number of z-stack +1 and "start z" = 0. However, this option may lead to faulty stitching and we recommend completing this procedure with the following steps.

vii. Immediately after starting, the stitching parameters and coordinates of the tiles will be saved in an automatically created file called "TileConfiguration_{zzz}.txt.registered" in the input folder. This file will be used to stitch the other channels as well (**Supplementary Figure 8c**).

viii. Rename the automatically created file by deleting the ".registered" and change all tiles of the z-panel numbers back to 0000 and set the new channel number that you want to stitch (e.g C00_xyz-Table Z0100 into C01_xyz-Table Z0000). Do not change the coordinates. Save the file and move the file into a new folder for stitching another channel. Each channel requires its own .txt file with the same coordinates, only C0x changed (**Supplementary Figure 8c-d**).

ix. In Fiji, open the macro for stitching called "Stitching-old-just_txtFile.py" (available in http://discotechnologies.org/) click on "Run". Load the .txt file that was just created in the previous point, which contains the stitching parameters for each channel and input the "Number of images" (**Supplementary Figure 8e**).

x. Click "OK" and start stitching the first channel. Conduct the same steps for each channel. **OPTIONAL** Stitching can also be performed by other algorithms: for example using TeraStitcher[60] (v.1.10; https://abria.github.io/TeraStitcher/), especially for correction of the shifting of the tiles in all x-y-z directions.

xi. Now, to stitch different tiling scans (already stitched previously) together (for example to obtain the whole mouse from different tiling scans of single individual body parts) proceed by using Vision4D from Arivis (version 3.4.0) (**Supplementary Figure 9 and 10**). Vision4D does not require RAM for stitching.

xii. Rename the single .tiff files (for example that compose the ventral side of a whole body), which you previously obtained from stitching in Fiji, by using the multi-Rename tool of an orthodox file manager software such as "Total Commander". The name of each single .tiff image must indicate the information about the channel (e.g. C00, C01 etc.) and the position of the panel/image in z in 4 digits (e.g. for the channel C00, new name of the files: C00-Z0000.tif, C00-Z0001 and so on (**Supplementary Figure 9a**).

xiii. Save all the renamed images from different channels into the same folder (i.e. C00-C01-C02) (**Supplementary Figure 9a-last panel**).

xiv. Drag your files composed of all channels and z-stacks into the Vision4D software and proceed with the import steps. Press "Yes" when "Assume same structure for all files?" comes up (**Supplementary Figure 9b**). **OPTIONAL** Open the software called "Arivis converter" to convert all the renamed images of the scan folder into a single file in .sis Arivis format in case the application is occupied. Click on "add files".

xv. Select "custom import". Select the output folder and give a name to the file (e.g. GFPM34-dorsal.sis). **CRITICAL** All the images must be in the same format (for example either compressed or not compressed, 16-Bit and same number of z-panels). Then, click "more options" and match the target pixel type to the one of your .tif files (in our case 16-Bit integer). Click "OK" (**Supplementary Figure 9b**).

xvi. A new window called "manual import map" window will pop-up. Go to "Selection" and "pattern matching", to check whether Arivis comprehends which part of the image name tells the information about channel and which about the z-panel (**Supplementary Figure 9b**).

xvii. Check if the output name is correct and confirm the setting. Start the conversion.

xviii. Arivis/Arivis converter will create a .sis file of your scan. **CRITICAL** This .sis file must be saved in a final folder which must be located into the local drive of the computer. Do not save it in the network drive, as an interruption in the network would terminate the process.

xix. After the first image set is converted and imported, the second volume (e.g. the other side of the whole body) to be fused must be added. Drag the second folder into the software. The same prompter will pop-up. Assume same structure for all images, and follow the same steps as before with the exception of choosing a "New image set" instead of "New file". After the conversion and the import is finished with the second image set, make sure to save the .sis file. If you were using converter so far, start Vision4D and open the first .sis file you want to consider by clicking "File" → "open" or by double clicking on the .sis file. Then, to open the second .sis file that has to be stitched to the first one you have to import it as "New Image Set" in Vision4D. **CRITICAL** The second .sis file and all the others must match the pixel type of the original images by clicking in "Target Pixel Type" (e.g. 16-Bit). Make sure to have enough gigabytes in the local drive to later save the final stitched file. You must decide from the beginning how many channels to stitch. Extra channels cannot be added later.

xx. Set the correct pixel size in µm for each volume by going in "Data" → "pixel size" and do this for all the volumes. The system is flexible and it is not necessary to have same pixel size for all volumes.

xxi. In "Extra" → "Preferences" select the desired quality of rendering. If dealing with very big data, reducing the quality of rendering help increase the speed.

xxii. Adjust the brightness and contrast and scroll in 2D to take a better look of the data

xxiii. If necessary, flip one of the volumes to match the orientation of another volume, by clicking in "Data" → "Transformation Gallery" → "Flipping". In "Flipping Properties" you can choose to flip around different axis. For example, to stitch the dorsal scan with the ventral scan of the whole mouse, select "Flip X-Axis" + "Flip Z-Axis" for the dorsal volume. Wait until the Image Set is flipped and press "Save". This can take up to 1-1.5 days depending on the data size. After checking that the flipping has worked correctly in the multi view window, close the original

version that was not flipped. **CRITICAL** Create a .docx/.doc or a .txt file in which you will keep track of how you flipped the volumes and the order that the different volumes were stitched to each other. This record will be important in case you want to repeat the process.

xxiv. Using the 2D visualization modality, look for 3 landmarks in the different volumes. A landmark consists of a single pixel in a structure of the tissue that appears in both or more volumes that have to be stitched together. We are interested in getting the coordinates of this landmark/pixel. **CRITICAL** The landmarks should be structures deep into the tissue, for example bone cracks, holes, or junctions. They should be fixed structures that do not move during the mounting of the sample and they should be as far as possible apart from each other in xyz. It is possible to flip the volumes as explained in the previous point to more easily find the same landmark in different volumes. We generally use bones and not internal organs as landmarks.

xxv. To highlight the selected landmark of interest, click on the icon "Place New Object/Marker" and click on the icon add "Marker" for each image set. Then, match the names of the corresponding landmarks through the "Show objects Table" (**Supplementary Figure 9c**).

xxvi. To get the xyz coordinates of the indicated landmarks, click on "Annotation" icon and go in "Properties" → "Marker locations". Record the landmarks and their coordinates in the .docx or .txt file (in case you want to re-use them in the future). You can rename each landmark appearing in all volumes using the same name (e.g. LM1). Save after each step.

xxvii. Click on "Data" → "Volume Fusion". Here, indicate which volume you want to specify as "Base image Set" and which volume to specify as "Moving Image Set". During stitching, the coordinates of the "Base Image Set" will be kept fixed, while the coordinates of the "Moving Image Set" will be changed to be able to transform the whole "Moving Image Set" in order to stitch it to the Base Image Set. We normally keep the ventral scan as "Base Image Set" and we indicate the flipped dorsal scan as "Moving Image Set" (**Supplementary Figure 10a**).

xxviii. Save it as new file and select "10% of Scale" (at this stage we do a first preview by scaling down) and give a name to the volume that will result from stitching.

xxix. In "Transformation" → "Landmark Registration" window "Add all annotations as Landmarks" for both "Base Image Set" and "Moving Image Set". By doing so, the list of landmarks will appear as 2 lists in this window. **CRITICAL** Make sure that the order of landmarks are the same in both image sets. Their locations are displayed in the volumes on the right side of the window (**Supplementary Figure 10a**).

xxx. Click run to automatically fuse the 2 Volumes/Image sets. This step will take some minutes depending on the power of the computer.

xxxi. After fusing, a new .sis file will be created. Open it using Vision4D (without closing the previous one which was used to start the stitching) and check the quality of fusion by scrolling it in 2D, by moving it in 3D and by changing the brightness and contrast.

xxxii. If the result is satisfactory, go back to the previous Vision4D window that was used to set the landmarks. The preview window can be closed and deleted. **? TROUBLESHOOTING**

xxxiii. Set the scale to "100%". Click run. This process will take 1-2 days and will create a .sis file consisting of the volumes now stitched together.

xxxiv. Export the .sis file into a series of .tiff images using "tiff exporter" function of the software. This process will take a few hours, depending on the size of your data. **CRITICAL** Vision4D

names the exported .tiff files with the z-panel information before the channel information. Renaming might be necessary if a specific pattern of labeling is necessary for further analysis.

xxxv. Now it is possible to visualize, render and analyze this stitched image series in different software such as Fiji, Imaris and Amira or published algorithms such as ClearMap and NeuroGPS-Tree. Note that the total amount of this original exported whole-body data set can occupy terabytes of storage. We strongly recommend to perform lossless compression of the data first as described above, then running subsequent 3D visualizations.

Anticipated Results

vDISCO represents a powerful method to unbiasedly and systematically study biological phenomena in subcellular detail, by introducing a new histological approach which takes into account the necessity and also the complexity of histological investigation of a whole biological system such as a whole organ or even an entire body of adult mice.

By developing a simple, reproducible, straightforward and rapid pipeline that combines different steps which have the purpose to decrease the tissue background, increase and stabilize the fluorescent signal and clear very large specimen, we were able to obtain high resolution 3D imaging data that could provide novel insight into an entire rodent.

vDISCO has been shown to be highly versatile as well, since it can be virtually implemented in the majority of labs because it doesn't require special equipment or skillset (**Figures 4,5 and Supplementary Figures 1,4**). In particular it can be applied in synergy with many different fluorescent imaging systems from epifluorescence microscopes, confocal microscopes to different kinds of light-sheet microscopes (**Figures 2,3,6-9 and Supplementary Figures 2,5-7**).

Previously, we have used vDISCO in mice to reconstruct the first high resolution whole-body neuronal map, investigate whole body neuronal and inflammatory changed after CNS damage and trauma, discover new anatomic structures such as short skull-meninges connections between skull marrow and meninges[23].

We have also used vDISCO to detect and localize all metastases at single-cell resolution in animal models for cancer: the data shown in **Figure 2** represent a typical imaging result obtained by vDISCO for a mouse bearing pancreatic cancer, where single metastatic cells are visible from the whole imaged body (while are not visible in bioluminescence imaging in **Figure 2a-b**). This kind of data has been shown to be highly crucial for analysis using AI algorithms which were able to provide the location, number and distribution of all cancer cells and the targeting of these cells by therapeutic antibodies[1].

Here, we also show the possibility of analyzing other biological structures that span from head to toe: **Figure 8** represents the first 3D reconstruction of the Prox-1+ lymphatic system where details of lymphatic vessels are visible in different organs such as lungs, intestine, thymus etc. Such data can become highly valuable for studies of whole body inflammatory responses.

The versatility of vDISCO is also demonstrated by the fact that it can be applied on single dissected organs composed by different kinds of tissues. This is achieved by using a simplified version of the method that relies on passive incubation of the nanobody (**Figures 6,7,9 and Supplementary Figure 3**). The application of vDISCO on dissected organs can significantly further simplify and speed up the whole immunolabeling procedure, while still yielding high quality data: for example, in **Figure 6** the distribution of monocytes/macrophages cells is visible in different organs and in **Figure 9d-h** all brain pericytes are visible with such detail that we can observe the single cells wrapping the brain vasculature.

Last, we display that vDISCO can be highly valuable in the virus tracing field: in **Figure 9a-c**, the increase and stabilization of the fluorescent signal expressed by viral vectors after vDISCO allows us to detect neurites including single axons (**Figure 9b,c**, red and cyan arrow-heads).

In conclusion, vDISCO is a method that offers the possibility to comprehensively analyze whole organs and bodies, hence, being an important tool for biomedical researchers to address a broad spectrum of scientific questions that require both single-cell and a systems-biology approach.

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Author contributions

A.E. and R.C. initiated the project. R.C. and C.P. developed the original vDISCO method. R.C. designed the experiments. R.C. and Z.I.K. performed most of the experiments. C.P. provided data for the cancer mouse, H.M. and S.Z. provided data for the PDGFRb-EGFP pericytes labelled brains. F.F.V, M.M and T.-L. O. and F.H. provided mesoSPIM data. C.V. and J.V.G. provided the custom-made nanobodies anti GFP. D.K. imaged and M.I.T helped for stitching the *Prox1*-EGFP whole body scans. R.C. and Z.I.K. supervised the experiments. A.E. supervised the project. R.C and Z.I.K wrote the manuscript. All the authors edited the manuscript.

Competing financial interests

A.E., R.C., C.P. and S.Z. have filed a patent related to some of the technologies presented in this work.

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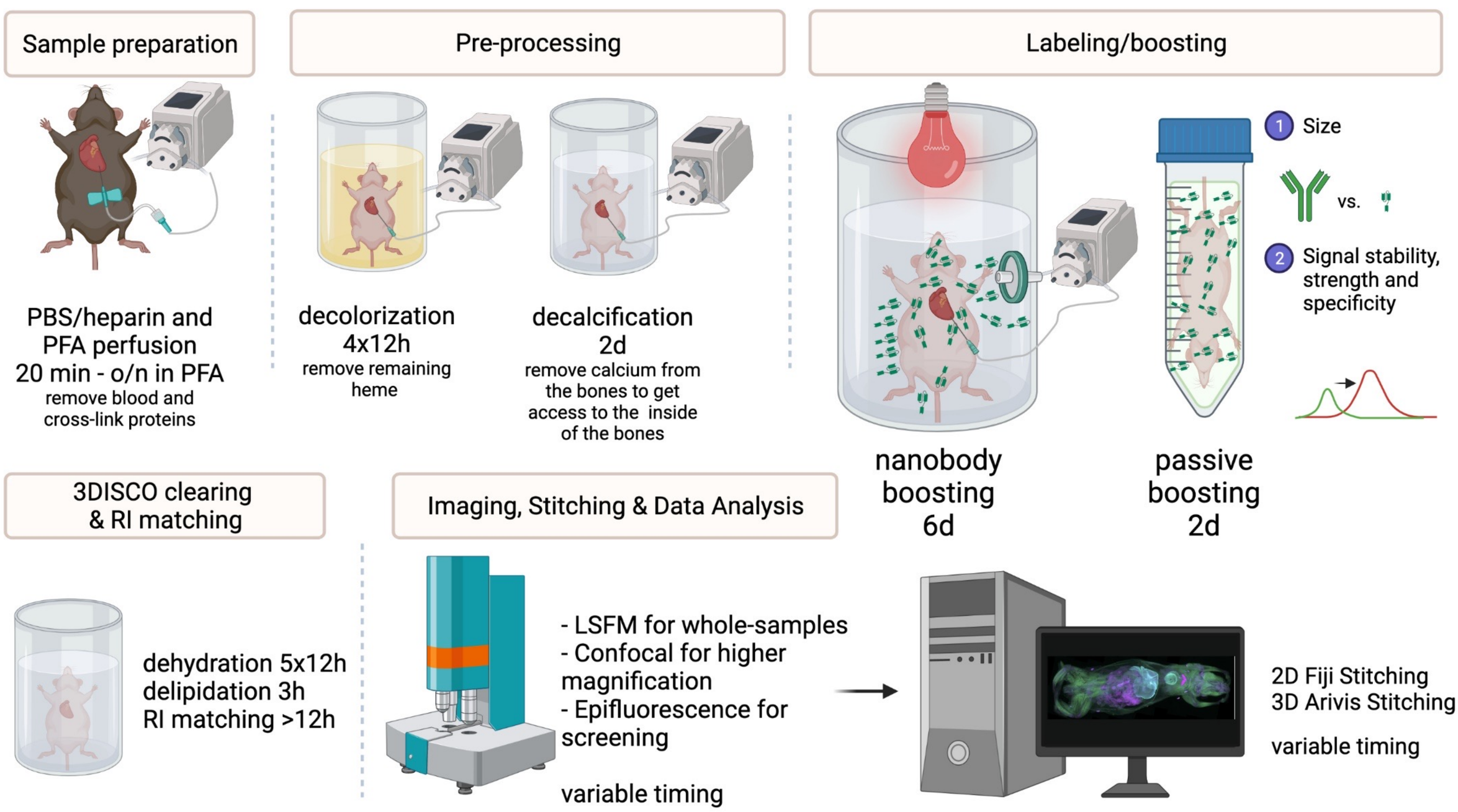


Figure 1

Overview of vDISCO pipeline

vDISCO protocol is composed of five main steps. After the sample is perfused with PBS and PFA, pre-processing steps such as decolorization and decalcification take about 4 days in total. Following this step, labeling and boosting take place, in which samples are incubated with nanobodies at higher temperatures for six days with active perfusion and two days in passive incubation. The use of nanobodies allows better penetration due to their small size, and better signal due to using long wavelength spectra. Additional dyes such as PI can also be incorporated here. Next the 3DISCO clearing is performed. As a result, the cleared sample can be imaged with different microscopes: light-sheet, confocal or epifluorescence depending on the research focus. After imaging, the data can be stitched if tiling scan was utilized and further analysis can take place. The whole procedure takes about 3 weeks and multiple samples can be processed at the same time.

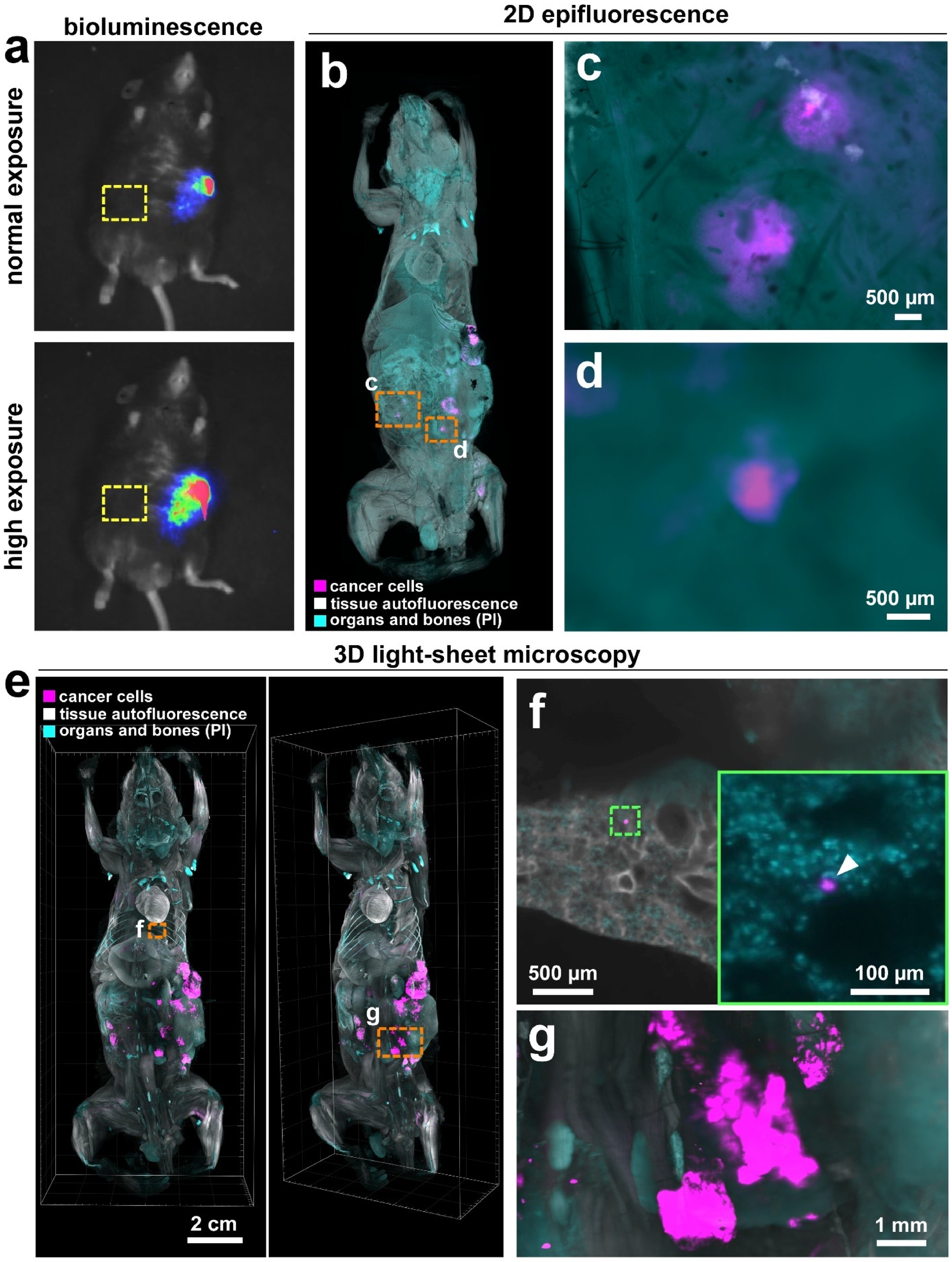


Figure 2

vDISCO imaging of a mouse with syngeneic pancreatic cancer.
(**a**) Normal and high exposure of bioluminescence imaging of the mouse with pancreatic cancer expressing eGFP at the end point of 38 days. (**b-d**) 2D epifluorescence microscope imaging of the same mouse after vDISCO labeling and clearing. The eGFP was boosted with nanobodies conjugated with Atto647N. (**c,d**) Zoomed in views of the boxed regions in b indicates that vDISCO pipeline provides more details of metastases (magenta) formation in peritoneum which is not available from standard bioluminescence imaging (see b-d v.s. a). (**e**) Ventral and 30° rotated views of the 3D reconstruction from light-sheet microscope scanning of the mouse. (**f**,**g**) Cellular level resolution light-sheet microscopy reveals individual metastases with various size and shape, including a single disseminated cancer cell detected in lungs (**f**, white arrow head). In b-g, pancreatic cancer cells are shown in magenta, organs and bones labeled by Propidium Iodide (PI) are shown in cyan, tissue autofluorescence is in white. Note that the f panel was obtained by imaging the animal 2 years after vDISCO processing.

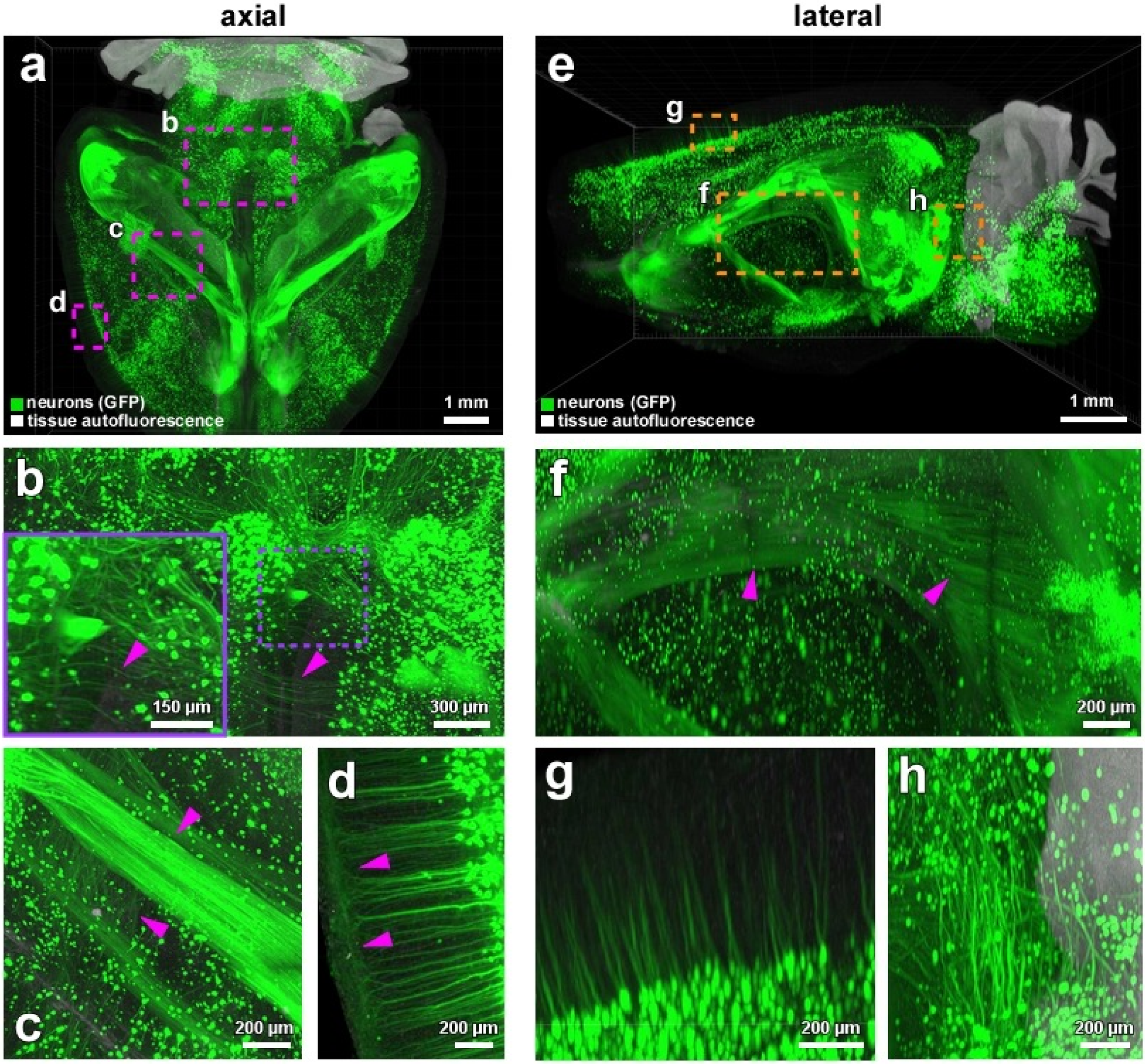


Figure 3

2 year old vDISCO processed *Thy1*-GFPM brain imaged with Lightsheet Z.1 microscope

Imaris 3D reconstruction of a *Thy1*-GFPM brain imaged by the Lightsheet Z.1 microscope 2 years after vDISCO processing. The brain was imaged in axial view and it is here shown in axial (**a-d**) and lateral view (**e-h**). (**b-d**) Zoom-in images of the areas indicated by the boxed regions in a, showing commissural axons (**b**, arrow-heads) crossing the two brain hemispheres, bundles of corpus callosum axons (**c**, arrowheads) and details of neurites of pyramidal cortical neurons projecting onto the surface of the brain (**d**, arrow-heads). (**f-h**) zoom-in of the areas indicated by the boxed regions in e, showing neuronal details after rotating the brain to lateral view (**e**): bundles of corpus callosum axons (**f**, arrow-heads), pyramidal cortical neurons (**g**) and commisural axons (**h**) are visible with continuity even in lateral view because of the isotropic resolution of the microscope. Similar results were achieved at least in 2 different samples.

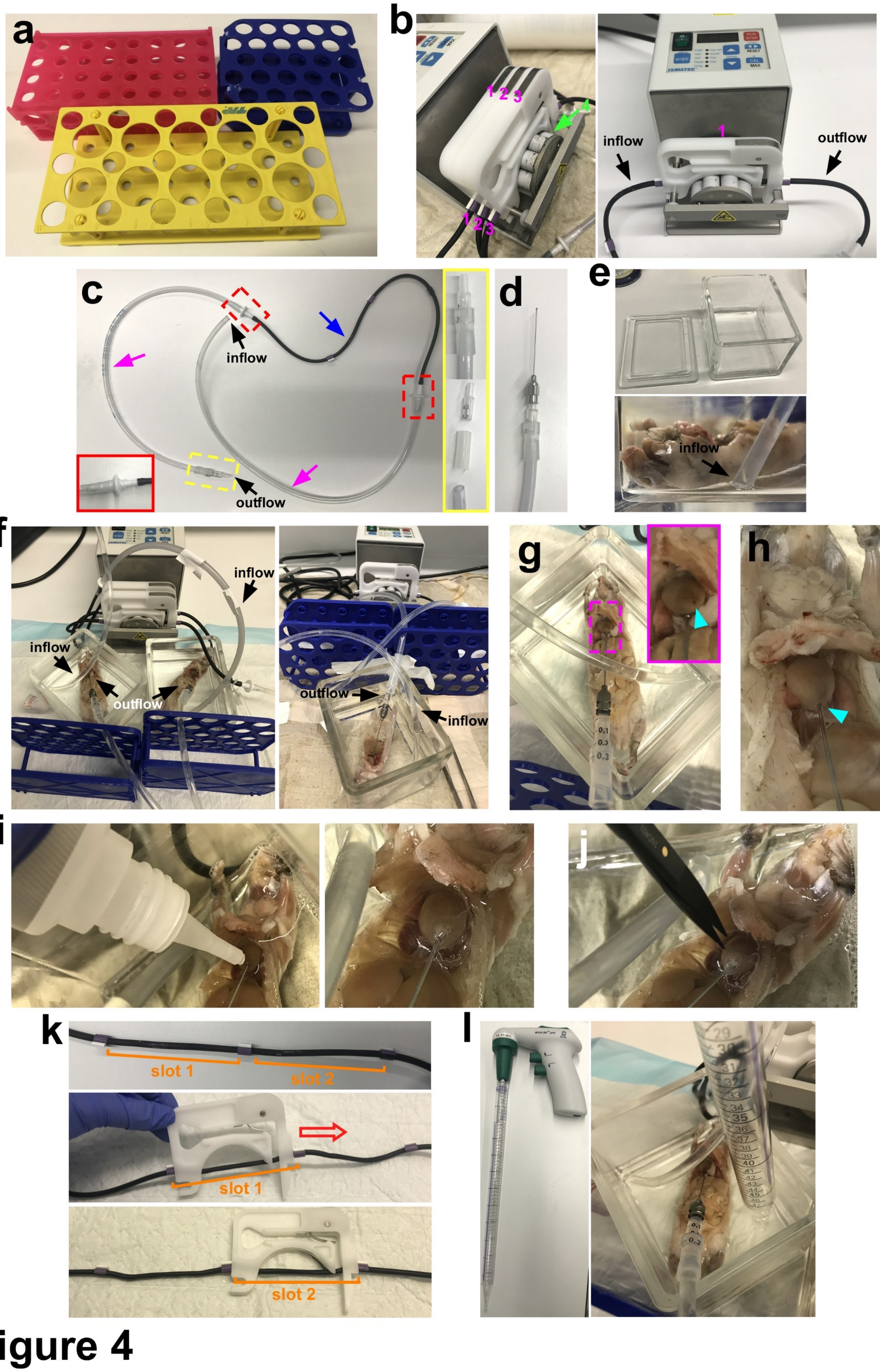


Figure 4

Whole body active-vDISCO set up

(**a**) Sample holders used in whole body active-vDISCO to hold the perfusion needle. (**b**) Peristaltic pump that can hold 4 pumping channels, therefore can process 4 bodies at the same time: in the left panel 3 channels are indicated by magenta numbers, the green arrow indicates the slot for the 4th channel; the right panel shows only one channel for the sake of simplicity: the inflow (sucking) end and the outflow (pumping) end are indicated by black arrows. (**c**) Components of the tubing for a single channel: one reference tube (blue arrow), two PVC tubes to extend the reference tubing (magenta arrows), two hose tubing connectors (red boxes), an outflow end made from the tip of 1 ml Braun syringe (yellow boxes). (**d**) Perfusion needle. (**e**) Glass chamber used for the perfusion and the whole body clearing steps (top); position of the sucking end of the pumping tube inside the glass chamber with the tip which reaches the bottom of the chamber (bottom). (**f**) Different ways to position the pump in relation to the samples and to the sample holders. The inflow and outflow tubes are indicated by black arrows. (**g-h**) The perfusion needle (**g**) inserted inside the entry point hole (cyan arrow) on the left ventricle. (**i**) Super glue is applied to seal the hole. (**j**) Cutting of the right ventricle. (**k**) Pumping slots of a single reference tube. (**l**) Strategy to change the perfusion solution.

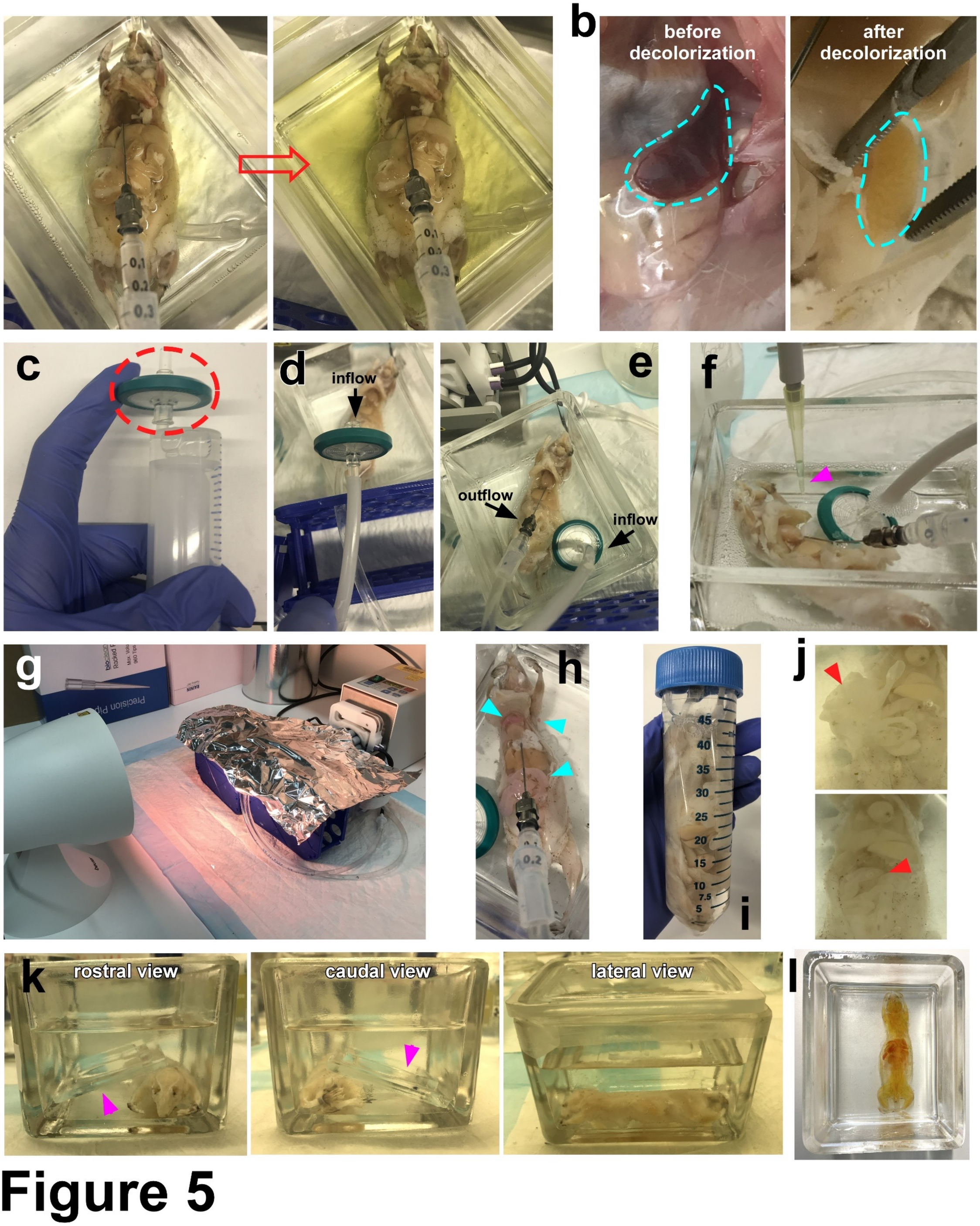


Figure 5

Whole body active-vDISCO procedure

(**a-b**) Pictures showing the successful elution of the heme from the body, indicated by the change of color of the decolorization solution which turns from colorless into yellow (**a**) and the change of color of some organs such as the spleen, which turn from red into beige (**b**). (**c**) The 0.22 µm filter (red dashed circle) used to prevent the formation of dye aggregates, must be wet by using a syringe before connecting it to the tube. (**d**) Mounting of the filter onto the inflow (sucking) end of the perfusion channel. (**e**) The inflow end with the filter is positioned inside the glass chamber. (**f**) Picture showing the adding of the dye into the staining solution (magenta arrow-head) using a pipette. (**g**) The chamber is covered with aluminum foil and heated up with an infrared lamp. (**h**) Picture showing some organs turning pink after PI staining (cyan arrow-heads). (**i**) Final passive staining of the whole body inside a tube. (**j**) Intestine is pushed into the abdomen before clearing. (**k**) Different views of the animal inside the glass chamber during the clearing step showing a glass lid positioned onto the animal (magenta arrow-heads) (**l**) Dorsal view of the cleared animal inside the glass chamber.

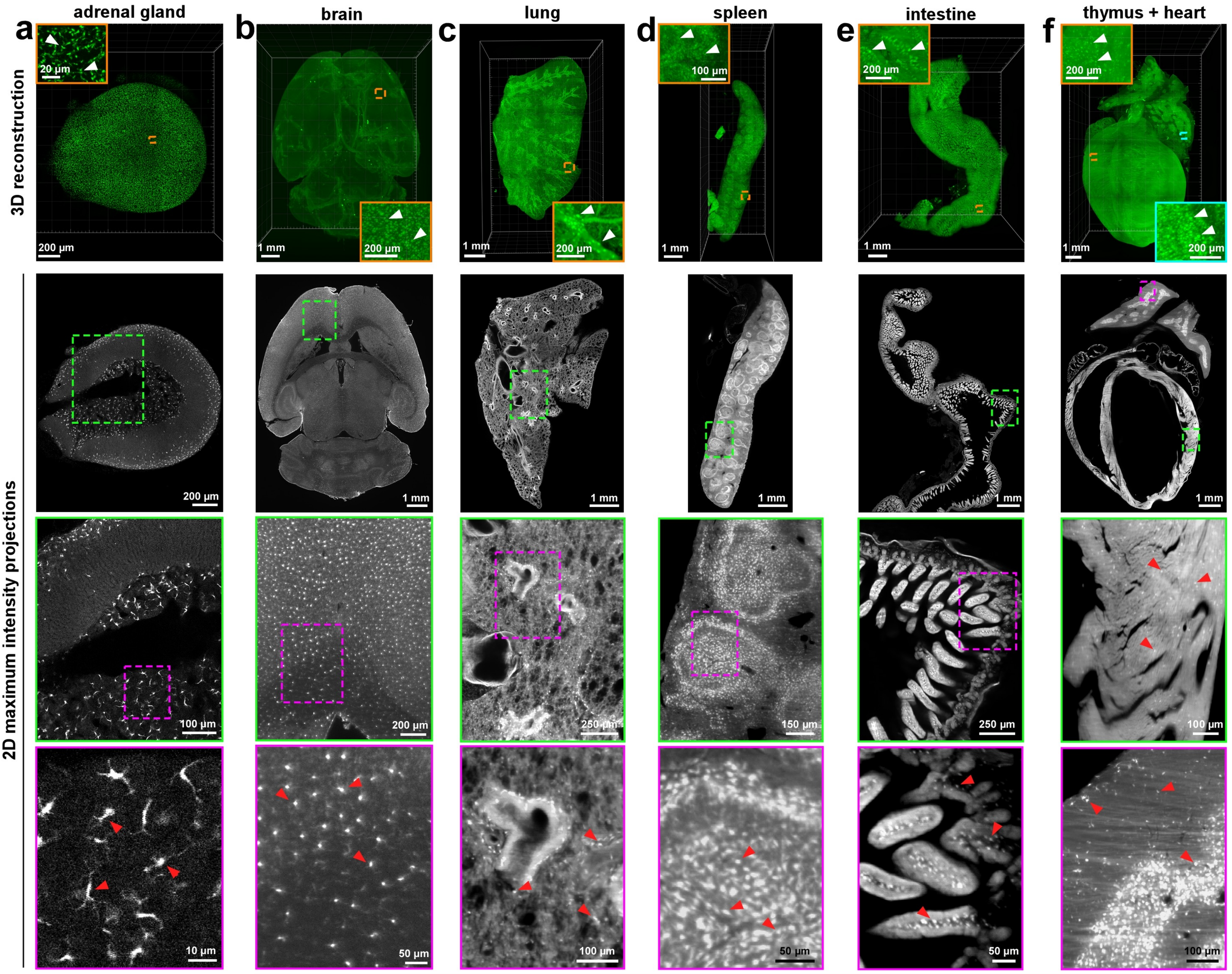


Figure 6

Passive-vDISCO on dissected organs imaged with confocal and light-sheet microscopy

3D reconstructions and 2D two-slices maximum intensity projections of an adrenal gland (**a**), dissected brain (**b**), lung lobe (**c**), spleen (**d**), intestine segment (**e**) and thymus + heart (**f**) coming from the CX3CR1$^{GFP/+}$ mouse line after passive-vDISCO. Zoom-in images from the dashed regions show individual CX3CR1 GFP+ immune cells (red arrow-heads). To remove residual blood, the organs in c and d were initially decolorized for 2 days in the decolorization solution (25% dilution of the stock), while the ones in e and f for 1 day in the decolorization solution (20% dilution of the stock). Similar results were observed in 2 independent animals.

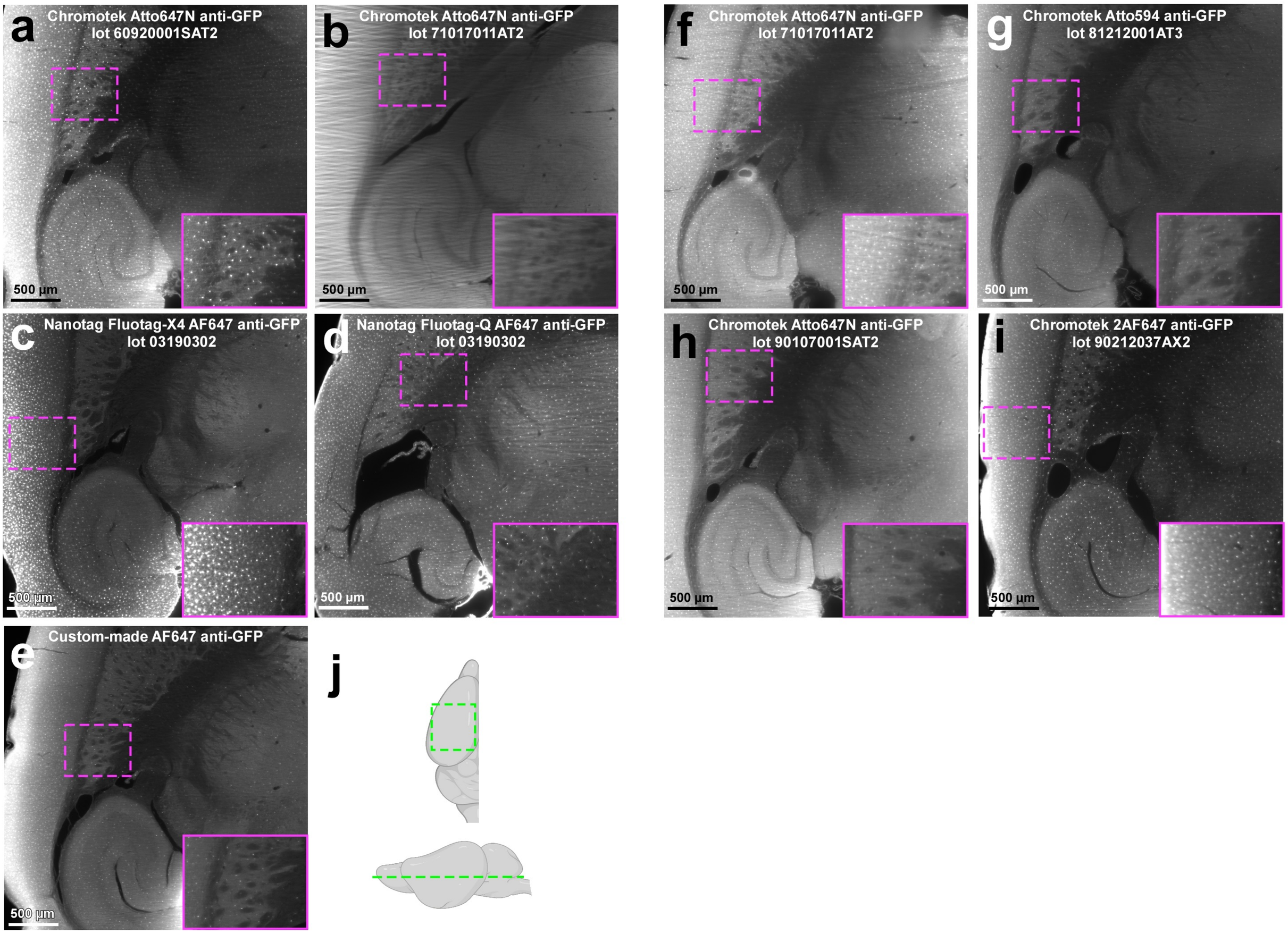


Figure 7

Performances of different kinds of nanobodies in passive-vDISCO

Light-sheet microscopy images of half mouse brains from CX3CR1$^{GFP/+}$ line showing the performances of different batches of nanobodies from different sources and companies using standard passive-vDISCO (**a-e**) and mild passive-vDISCO (**f-i**). The nanobody which was used is indicated in each panel title. (**j**) Illustration indicating the anatomic region of the brain (green dashed) that is displayed in a-i. Note that the imaging was taken either on the right or on the left hemisphere, the right hemisphere images were flipped to ease the comparison between the different nanobodies. (All the results in this figure were similarly observed in at least 2 independent experiments for each kind of nanobody).

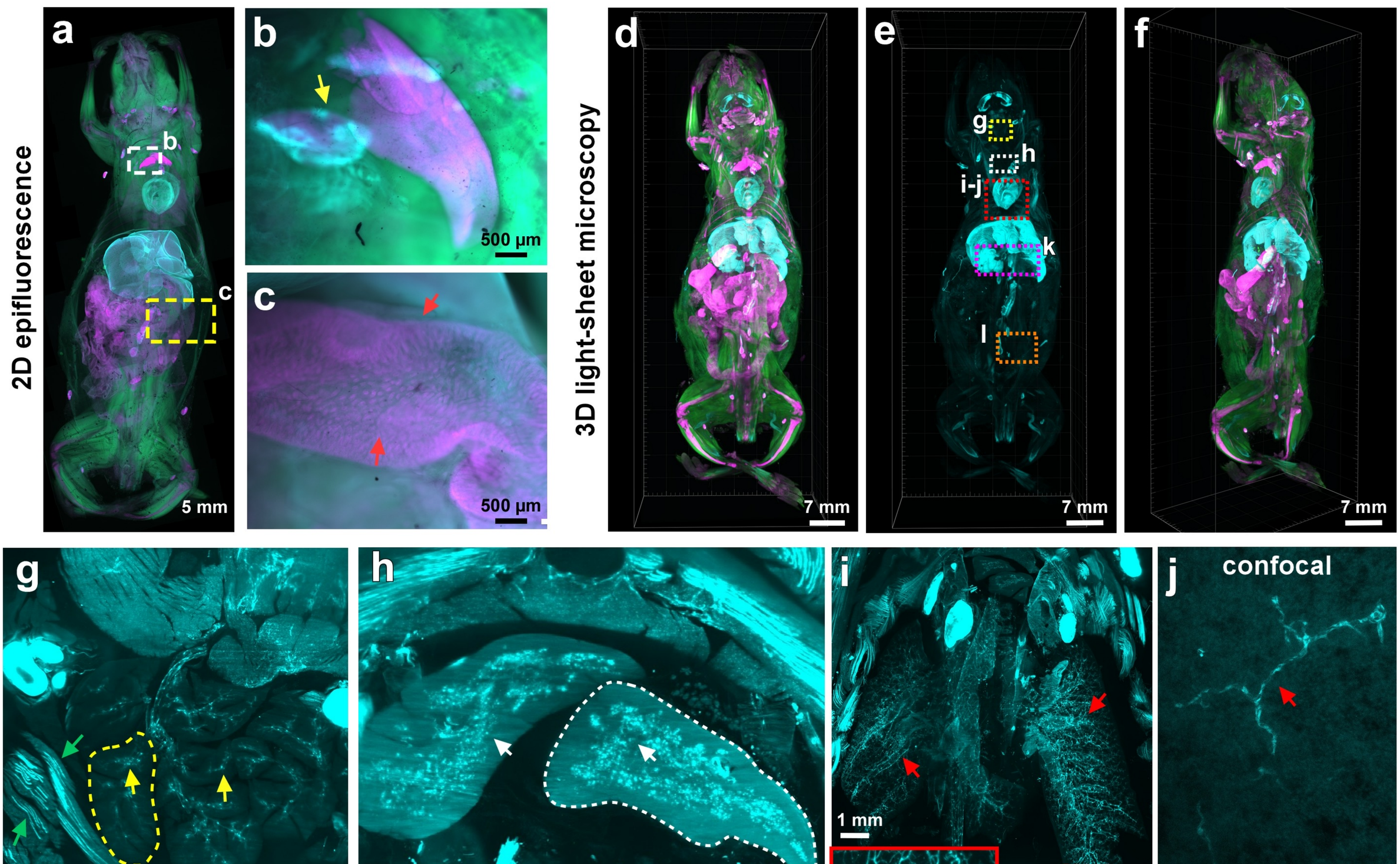


Figure 8

Prox1-eGFP whole-body scan taken with epifluorescence, light-sheet and confocal microscopy

(**a-c**) 2D epifluorescence microscope imaging of a *Prox1*-eGFP mouse after vDISCO pipeline. (**b,c**) Zoomed in views of the dashed regions in a showing the thymus (yellow-arrow) and the intestine, respectively. Red arrows in c indicates intestine villi. (**d-f**) 3D reconstruction of the same mouse in a imaged with light-sheet microscopy in dorsal (**d,e**) and 30° rotated view (**f**). (**g-i** and **k,l**) High magnification images of the different body regions indicated by the dashed boxes in e from the same whole body light-sheet imaging scan in d. (**j**) Confocal imaging of the lungs in i. (**g,h**) *Prox1*-eGFP+ signal is visible as cells in the region including the salivary glands (**g,** yellow arrows) and thymus (**h,** white arrows). The dashed yellow and white lines in g and h delimit a salivary gland and a thymus lobe, respectively. (**g** and **i-l**) *Prox1*-eGFP+ signal is visible as elongated structures in the body regions including some muscles (**g, k** green arrows), in the body region showing the lungs (**i**,**j** red arrows), in the body region including the pancreas (dashed violet box in **k,** violet arrows), in the kidneys (**k,** magenta arrows, right kidney is delimited by the dashed magenta line) and in the intestine (**l**, orange arrows). g-i and l panels are shown in ventral view as k panel is shown in dorsal view. Note that some details of the *Prox1*-eGFP+ signal that are not visible in 2D epifluorescence imaging are now visible in light-sheet imaging, for example in the thymus (b v.s. h) and in the intestine (c v.s. l). *Prox1*-eGFP+ signal is shown in cyan in all the panels. In a-c and d,f organs and bones are labeled by Propidium Iodide (PI) and shown in magenta while tissue autofluorescence is shown in green.

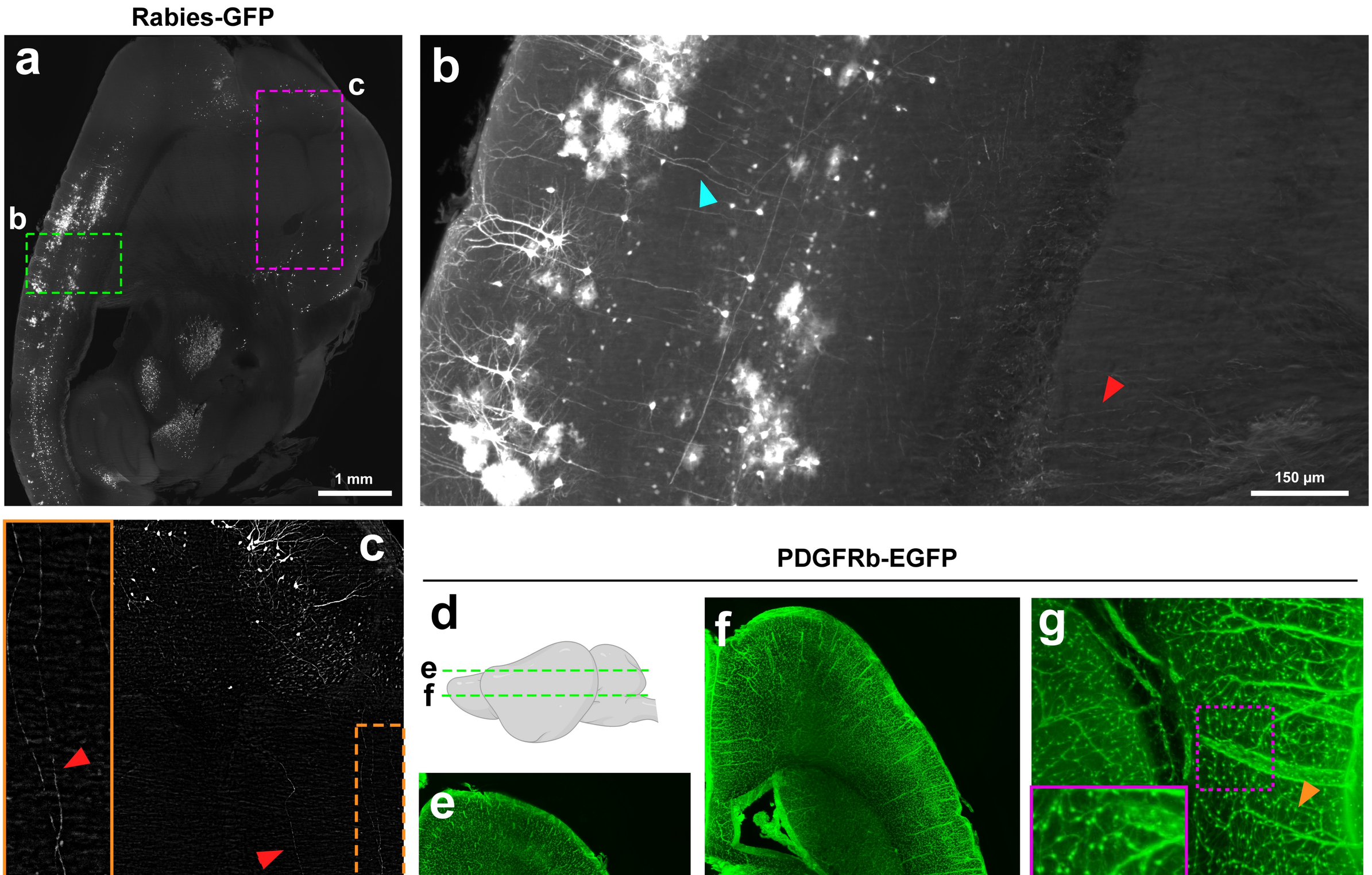
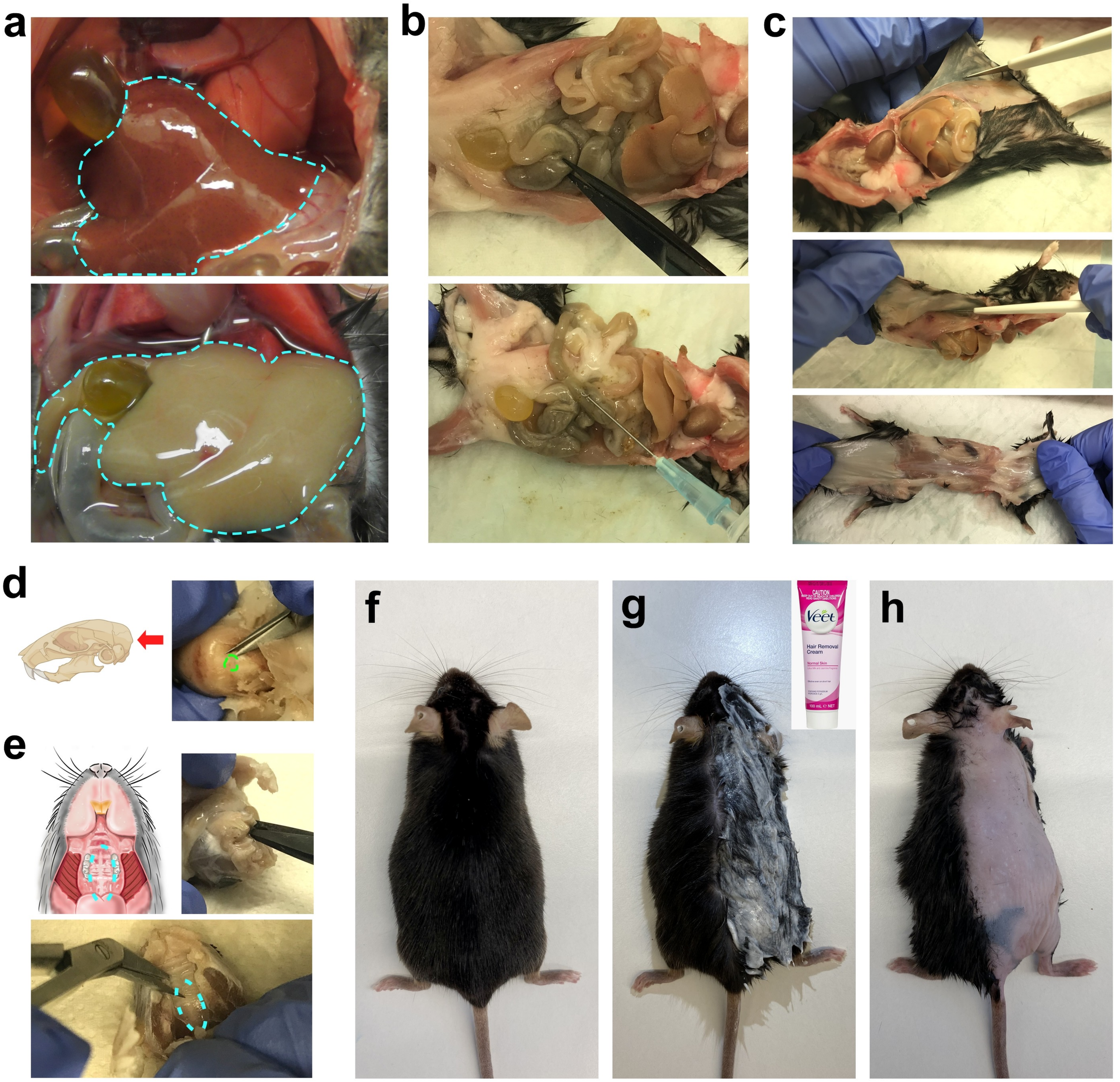


Figure 9

Virus tracing and pericytes in the brain processed by vDISCO

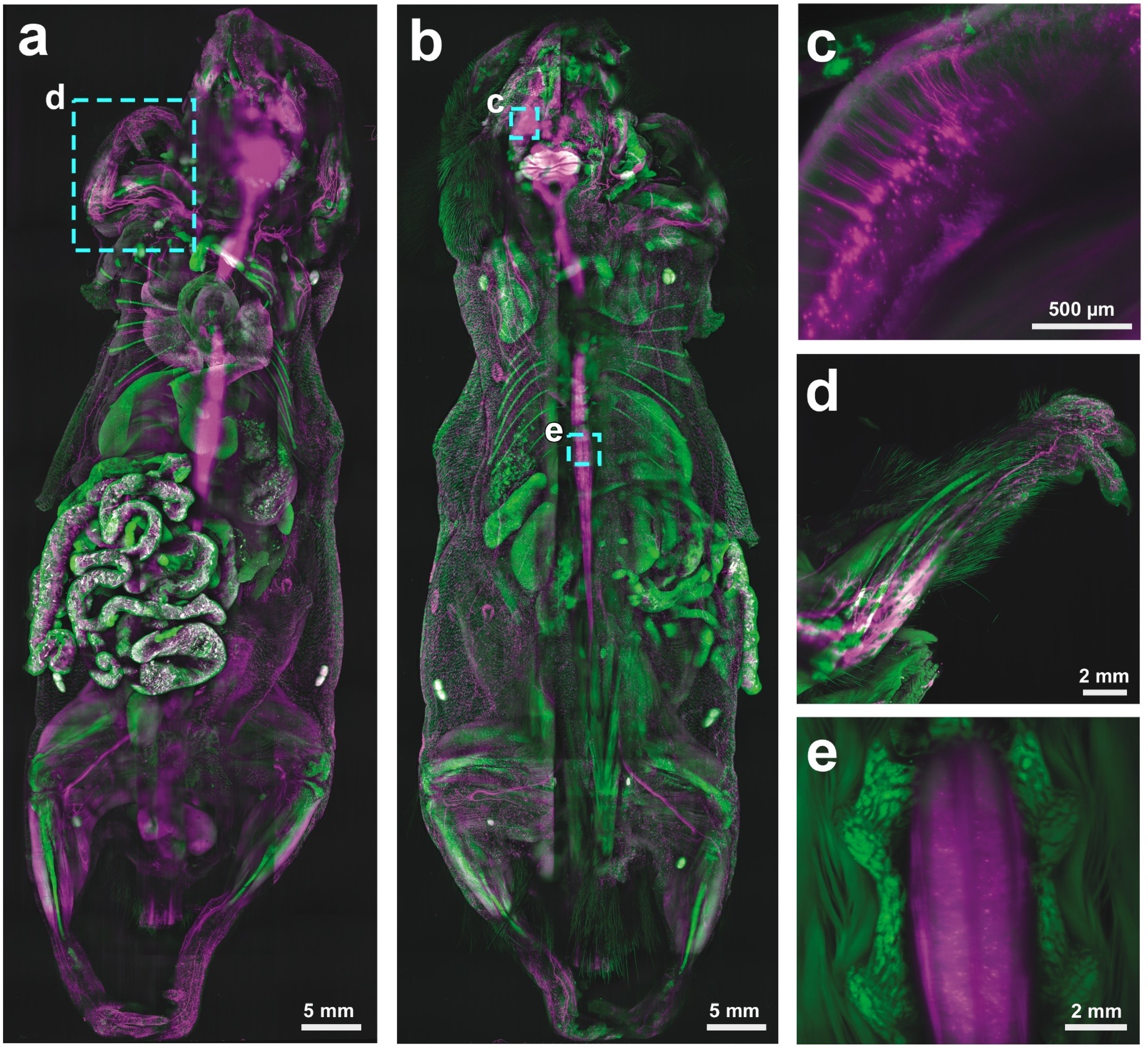
(**a-c**) Light-sheet microscope 2D images of a brain from an adult Emx1-Cre x RΦGT injected with EnvA-pseudotyped G-deleted rabies virus expressing GFP (SADB1969) into the neocortex and processed with vDISCO. The images show the virus traced neurons with single axons visible in the cortex (**b**, cyan arrow-head) and in the striatum (**b**, red arrow-head) in the raw data and in the post-processed image (**c**, red arrow-heads). The post-processing was performed with sharpening of the signal, nouse filtering and background equalization and removal. (**d-h**) Half brain of a PDGFRb-EGFP mouse processed with vDISCO and imaged with light-sheet microscopy (**e-g**) and confocal microscopy (**h**): 2 images (**e,f**) corresponding to optical slices indicated in d are shown. Single EGFP+ pericytes are visible throughout the brain and wrapping blood vessels (**f,g**, orange arrow-heads). (**h**) In confocal imaging the pericytes somas (orange arrow-heads) and their fine processes (white arrows) are visible. For both virus traced and pericyte samples, similar results were obtained at least in 3 independent brains.



Supplementary Figure 1

Sample preparation for whole body active-vDISCO

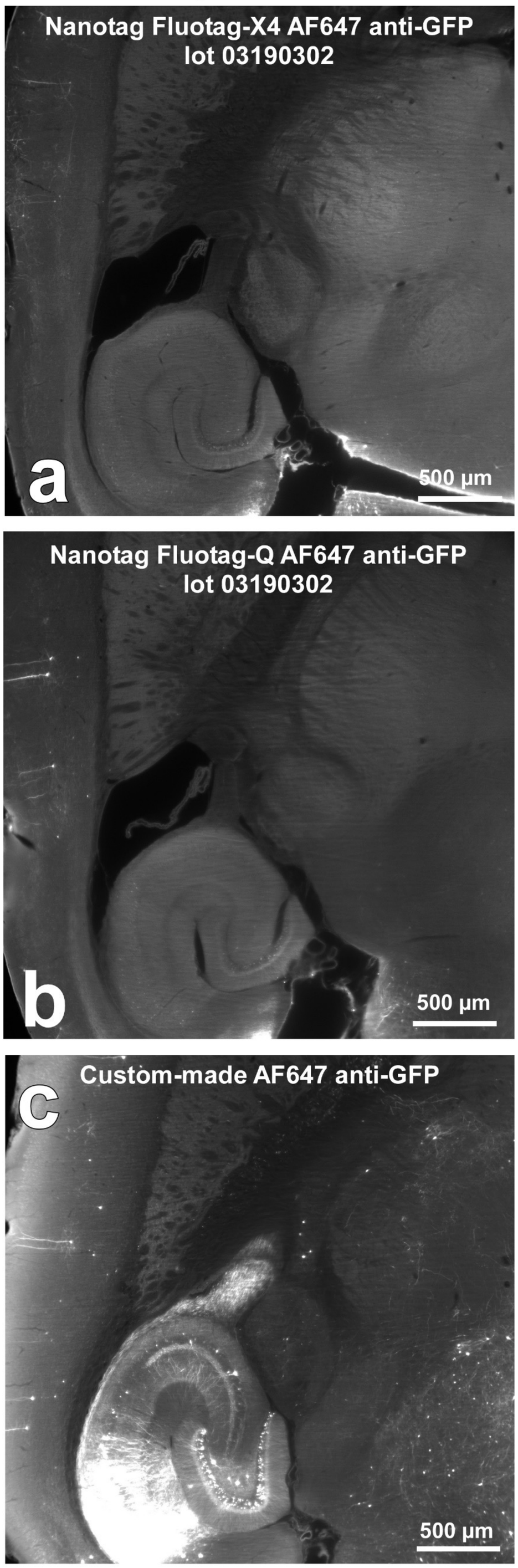
(**a**) During the 1x PBS perfusion step of the anesthetized animal, the color of the liver (cyan dashed line) turns yellow as indication of proper drainage of the blood from the body. (**b**) Some cuts in the gut are necessary to flush out the gut content and the feces with a syringe. (**c**) Pictures showing the procedure of skinning the body of the animal using a blade. (**d**-**e**) A piece of the occipital bone (back of the skull) indicated in d by the green dashed line and the hard palate indicated in e by the cyan dashed line must be removed after the PFA perfusion step. (**f-h**) In case the animal is needed with intact skin, commercial hair removal creams may be used as illustrated. The cream should be applied to region of interest and removed with water after 3-5 minutes. Steps can be repeated until the desired quality of hair removal is achieved.



Supplementary Figure 2

Whole-body imaging of a sample with intact skin using a modified mesoSPIM light-sheet microscope

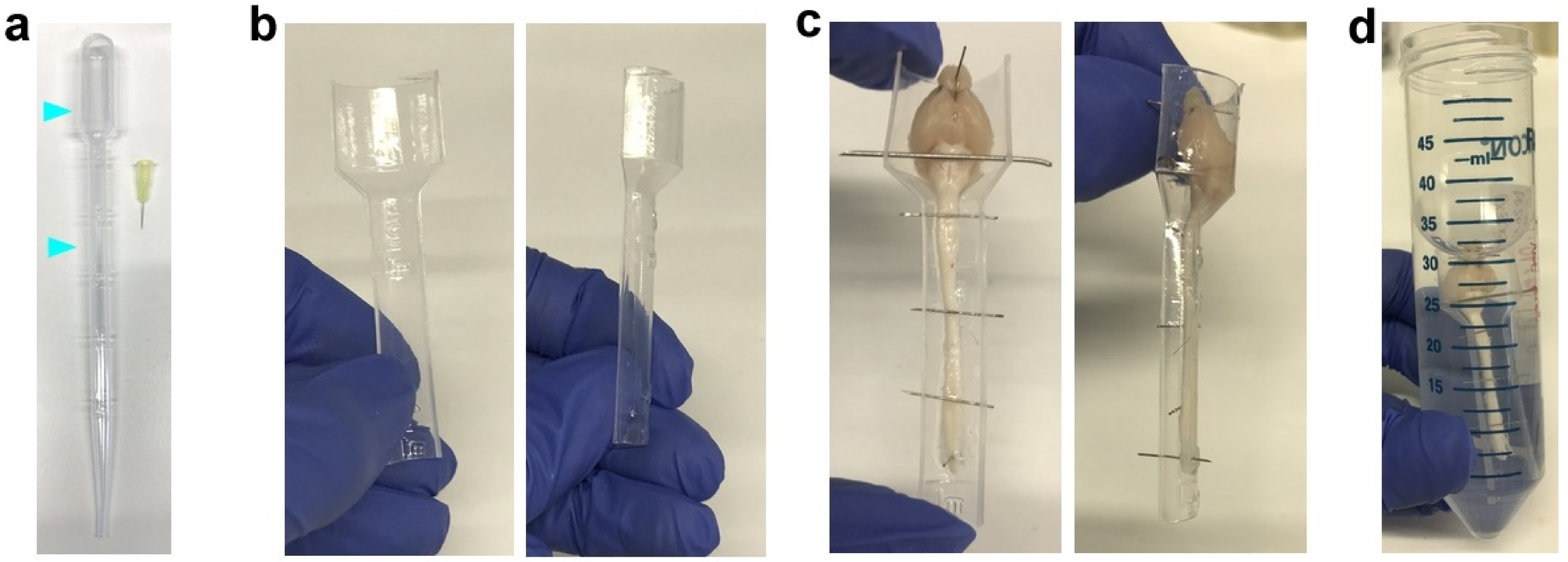
(**a**) Maximum projection of an adult *Thy1*-GFPM mouse with intact skin imaged with a modified mesoSPIM light-sheet microscope from the ventral side . Autofluorescence excited at 488 nm is shown in green, GFP in magenta. (**b**) Maximum projection of the same sample imaged from the dorsal side (rotated by 180°). (**c**) Layer 5 pyramidal neurons in the brain (**d**) Peripheral nerves and skin of the forepaw imaged from the lateral direction (90° rotation). (**e**) Detail of the spinal cord and vertebrae. Images in a, b, d and e were taken at 0.9x magnification whereas image in c was imaged at 4x magnification.



Supplementary Figure 3

Performances of different kinds of nanobodies in passive-vDISCO for *Thy1*-GFPM line

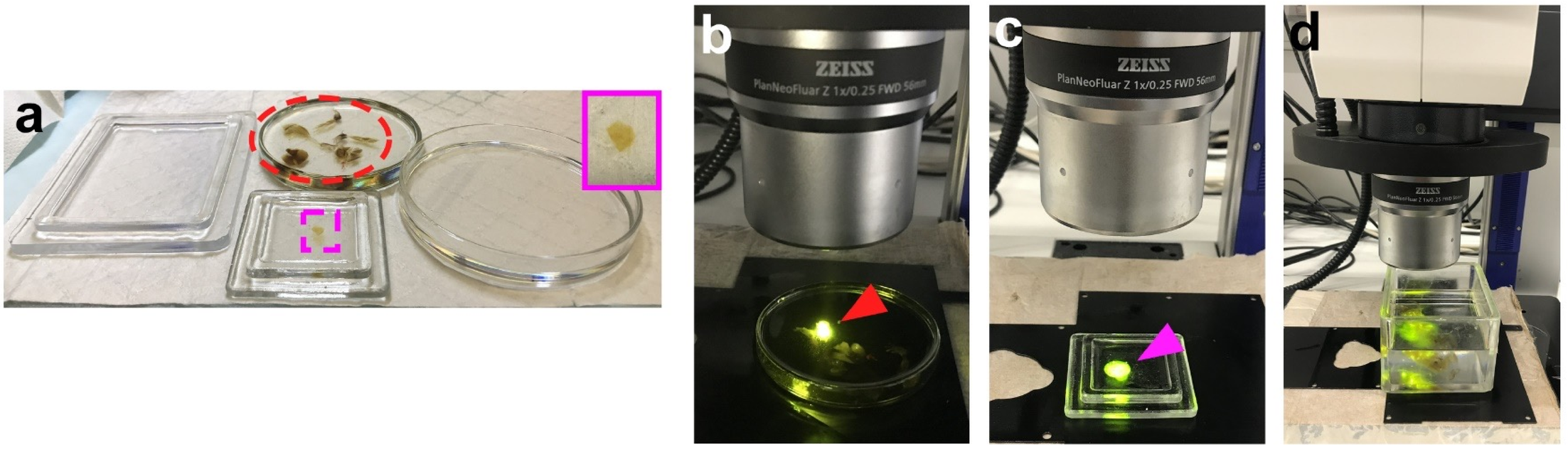
Light-sheet microscopy images of half mouse brains from *Thy1*-GFPM lines showing the performances of different batches of nanobodies from different sources and companies using standard passive vDISCO (**a-c**) and mild passive-vDISCO (**d,e**). The used nanobody is indicated in the panel title. (**f**) Illustration indicating the anatomic region of the brain (green dashed) that was displayed in a-e, note that the imaging was taken either on the right or on the left hemisphere, right hemisphere images were flipped to ease the comparison between the different nanobodies. (All the results in this figure were similarly observed in at least 2 independent experiment for each kind of nanobody).



Supplementary Figure 4

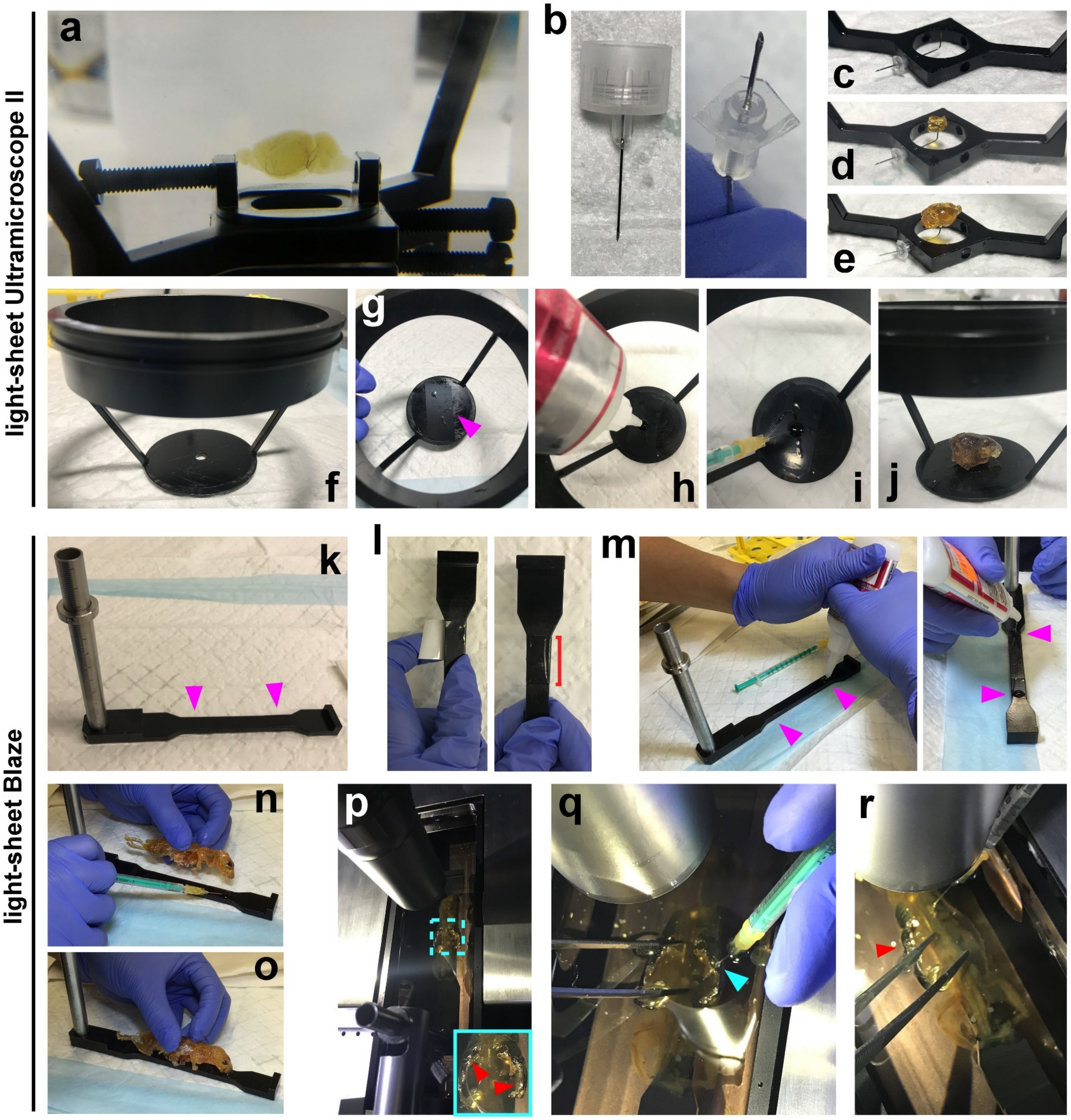
Strategy to make spinal cord straight for passive-vDISCO

(**a**) Required materials: a plastic Pasteur pipette and some fine needles. The cyan arrowheads indicate the cutting points. (**b**) The plastic Pasteur pipette is then longitudinally cut in half. (**c**) Positioning of the needles to constrain the brain with the spinal cord inside one of the halves of the pipette. (**d**) The whole setting is put into a container such as a 50ml tube for passive-vDISCO protocol.



Supplementary Figure 5

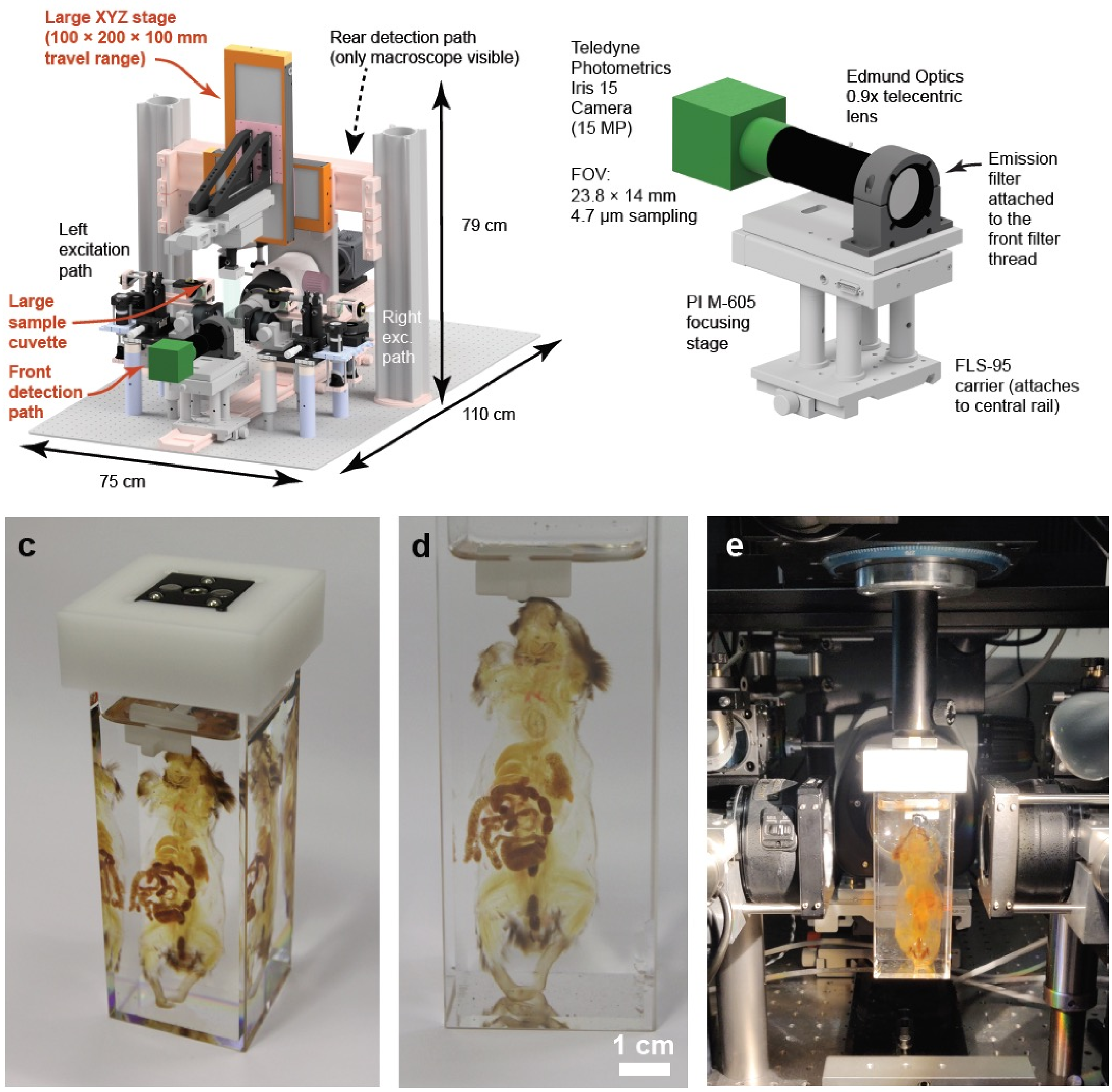
Mounting of cleared samples for epifluorescence imaging and inverted confocal imaging
(**a-d**) Mounting of different samples for AxioZoom epifluorescence imaging**.** (**a**) Different glass containers used to mount cleared organs (red dashed circle) and slices (magenta boxes) for AxioZoom epifluorescence imaging. (**b-c**) Epifluorescence imaging of dissected organs (red arrowhead) and slices (magenta arrow-head) with the AxioZoom microscope. (**d**) Epifluorescence imaging of the whole body with the AxioZoom microscope. (**e-g**) Mounting of different samples for inverted confocal microscope imaging: a slice (**e**) and a whole brain (**f**) are placed onto a glass bottom dish, then the dish with the lid is positioned onto the stage of the microscope (**g**)



Supplementary Figure 6

Mounting of cleared samples for light-sheet imaging

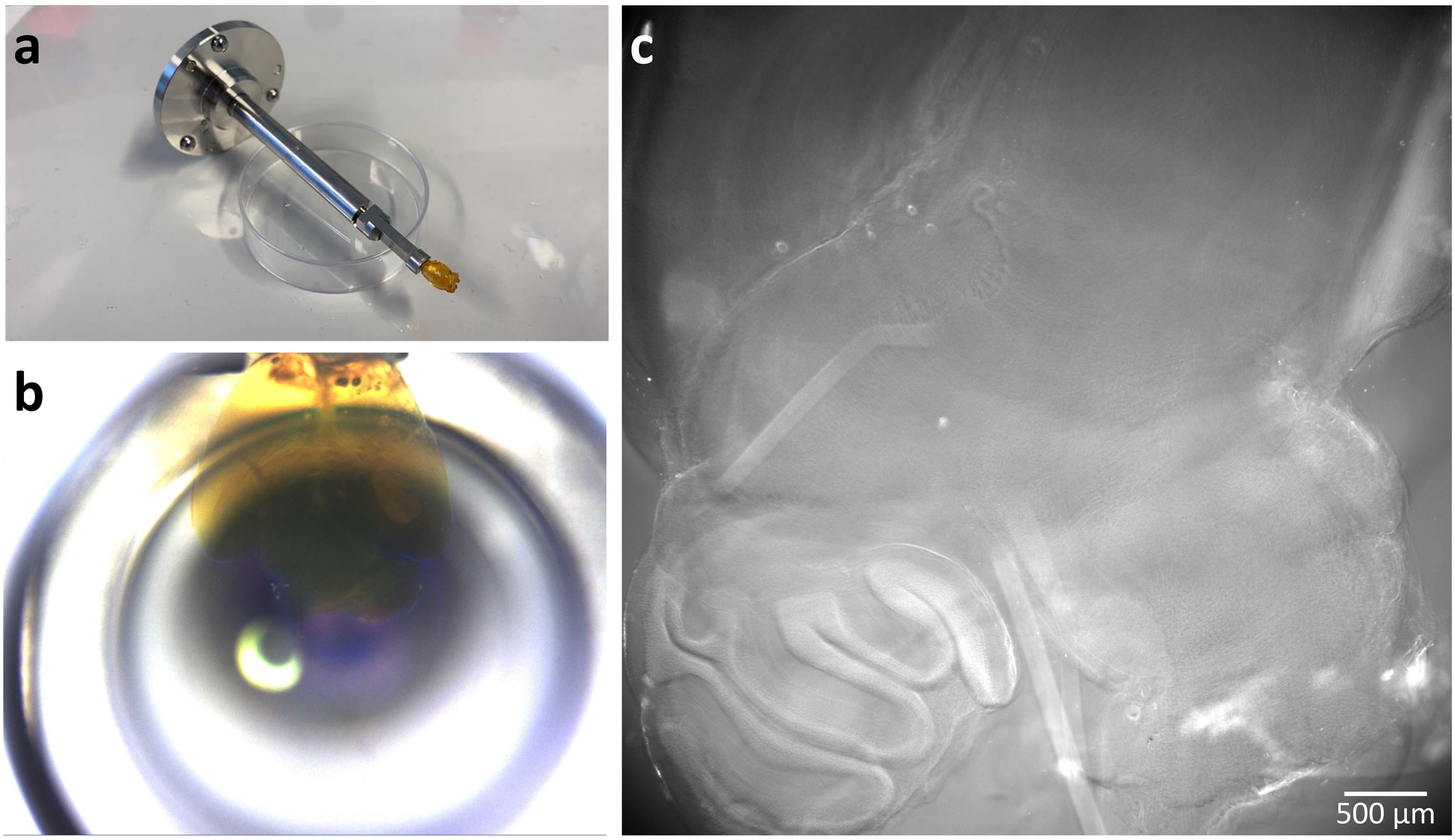
(**a-j**) Various strategies to mount different samples for LaVision-Miltenyi light-sheet Ultramicroscope II imaging. A whole brain is mounted using the screw system provided by the microscope supplier (**a**), a slice (**d**) and a whole brain (**e**) are mounted using Micro-Fine Ultra needles (**b**) attached to the sample holder (**c-e**). To mount a whole head using a flat sample holder (**f**), a piece of black tape is sticked to the flat surface (**g,** magenta arrow-head), then by adding super glue (**h**) and accelerator (**i**) the head is stabilized onto the tape (**j**). (**k-o**) Mounting of a whole body sample for light-sheet imaging using the Blaze microscope: two pieces of black tape are sticked onto the mounting region of the samples holder (**k,l**, magenta arrow-heads), superglue is applied onto the black tapes (**m**, magenta arrow-heads), accelerator is injected into the superglue (**n**) and the animal is positioned onto the sample holder at the level of the tapes (**o**). (**p-r**) Strategy to remove air bubbles (**p,r**, red arrow-heads) from the samples (e.g. a whole body) by inserting a fine needle into the bubble (**q**, cyan arrow-head) and sucking the bubble out with the syringe (**r**).



Supplementary Figure 7

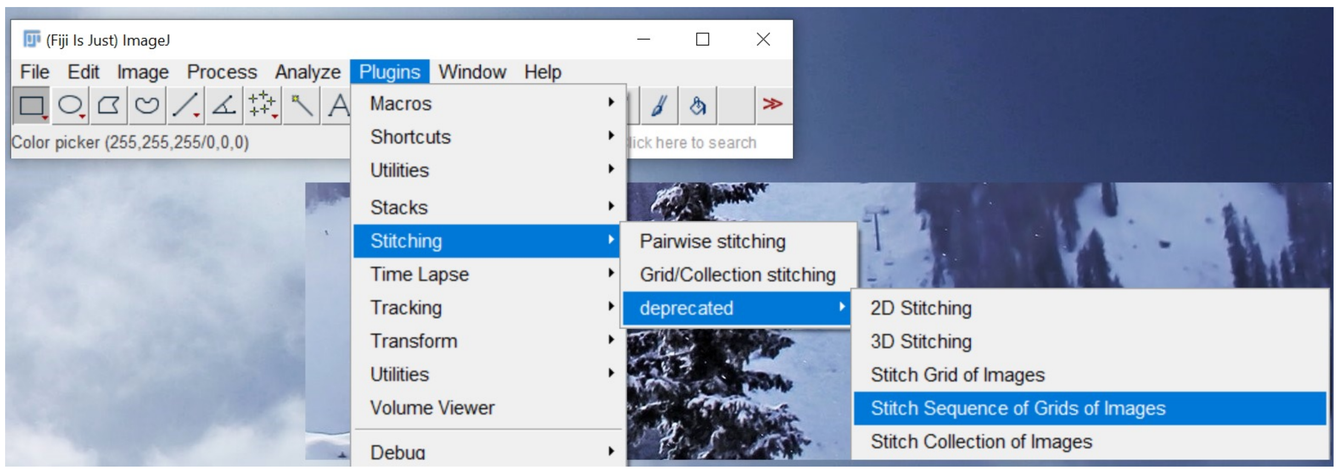
Whole body imaging with a mesoSPIM: setup modifications and sample handling

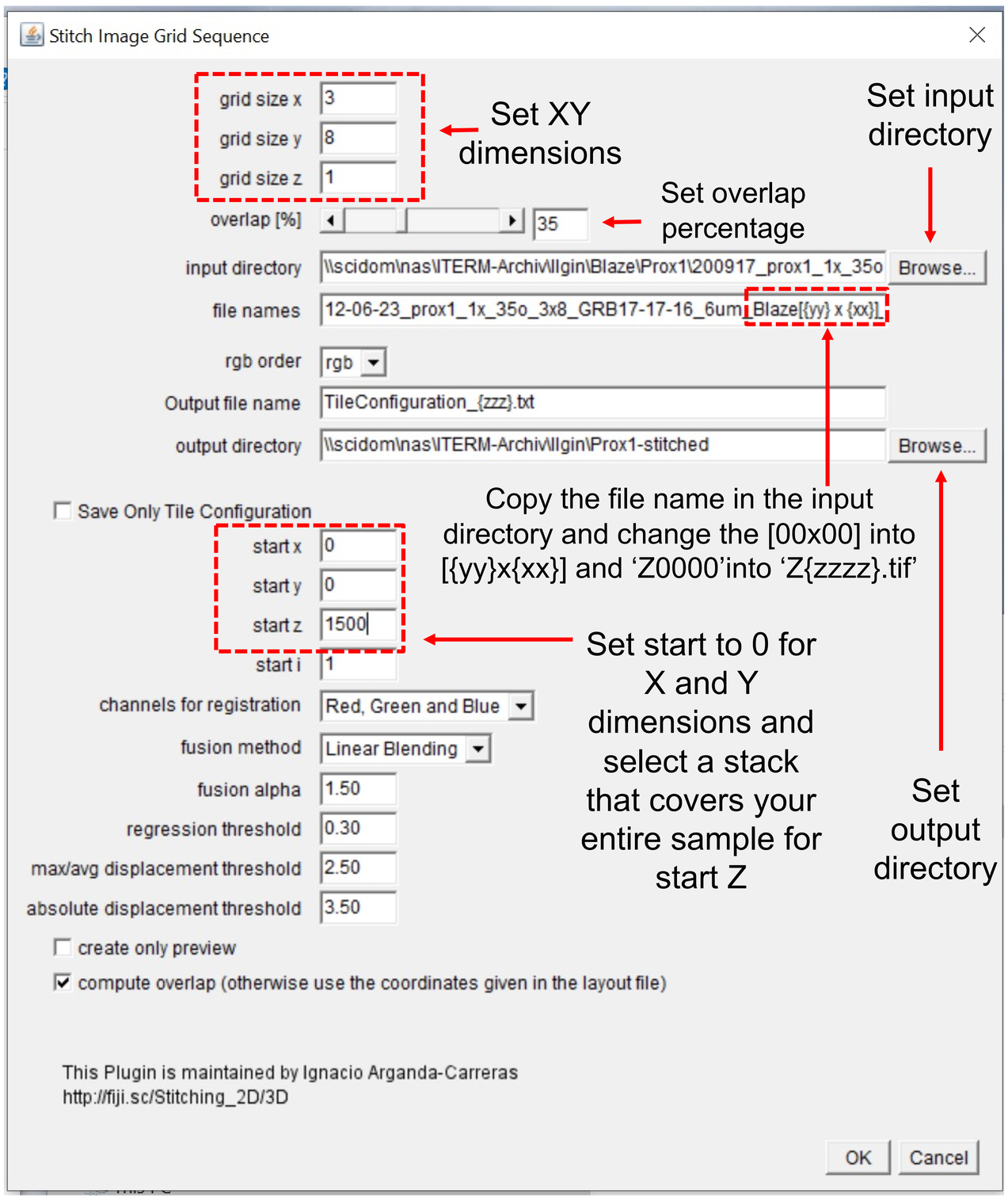
(a) Overview of the modified mesoSPIM setup: An existing mesoSPIM was modified by replacing the sample xyz translation stages with stages with larger travel range and by adding a second detection path in the front of the setup. **(b)** Design of the modified detection path with a telecentric detection lens with fixed magnification and a camera with high pixel count. **(c)** The sample was mounted in a custom 40x40x120 mm^3 quartz cuvette. The cuvette was closed with a custom lid which includes a kinematic mount with magnets which attach to the XYZ stages. **(d)** To stabilise the sample inside the cuvette, a 3D-printed crossbar with 40 mm length was inserted above the sample and gently pressed down. **(e)** View of the sample between the mesoSPIM excitation lenses before the front detection path was inserted.

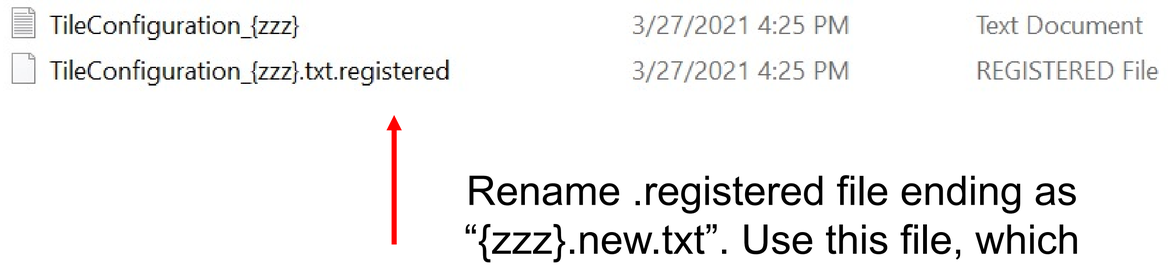


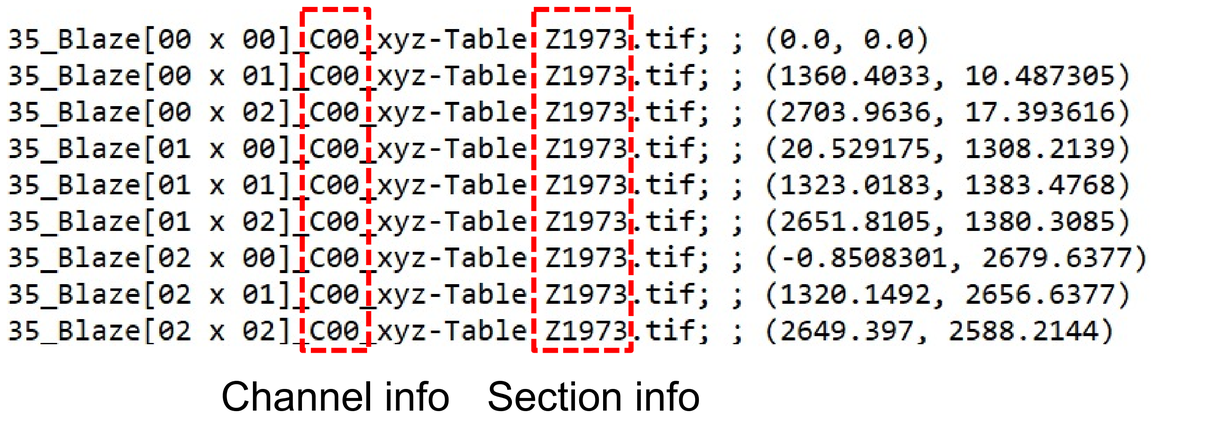
Supplementary Figure 8
Whole-brain imaging with Zeiss Lightsheet Z.1

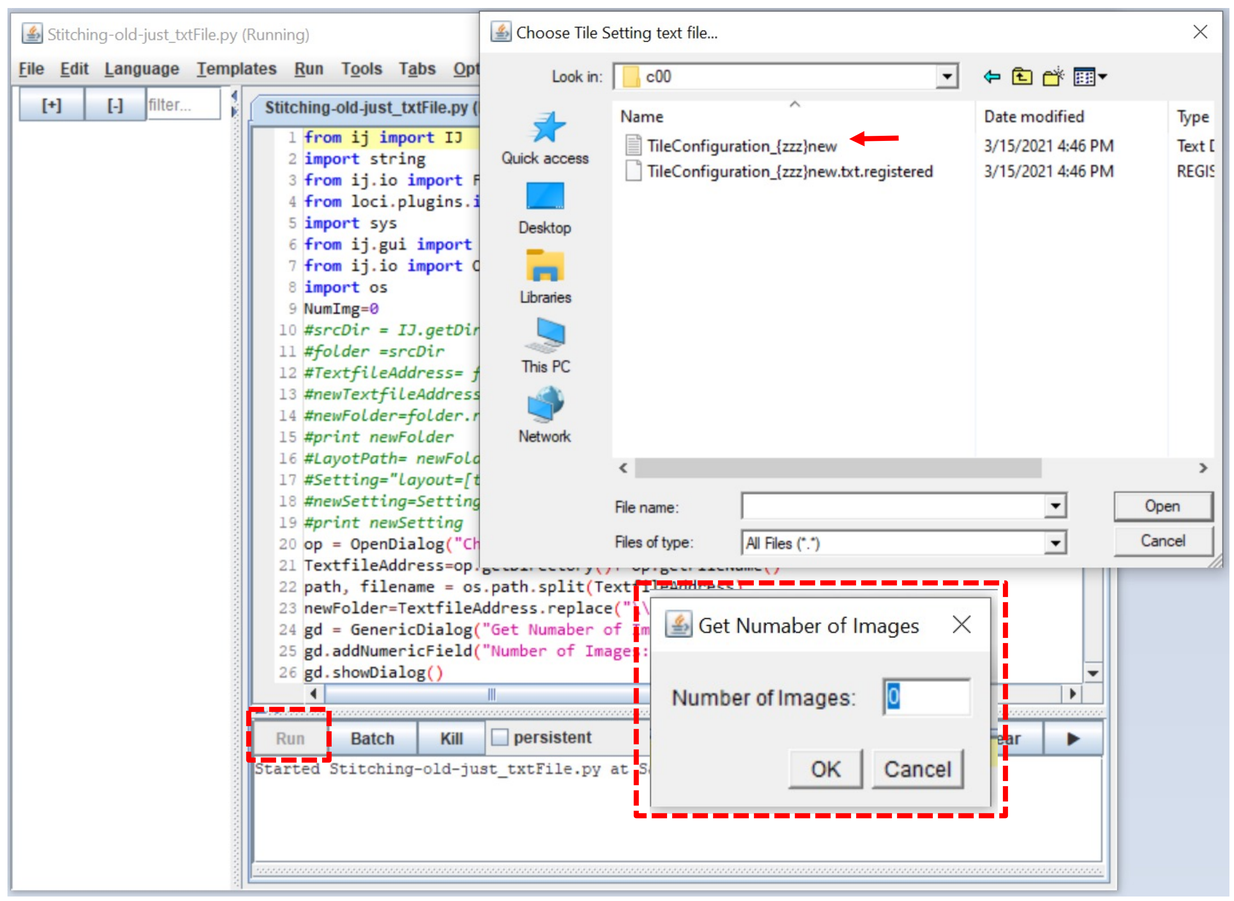
(a) The cleared brain sample is glued to the Lightsheet Z.1 rotatatable sample holder. **(b)** After mounting the sample holder into the microscope and inserting the imaging chamber containing the clearing solution, the sample is positioned in front of the detection objective. The right position in X, Y, Z and the rotation angle are monitored via the door camera of the microscope. **(c)** The focus plane is adjusted using Near-IR pseudo-bright-field illumination







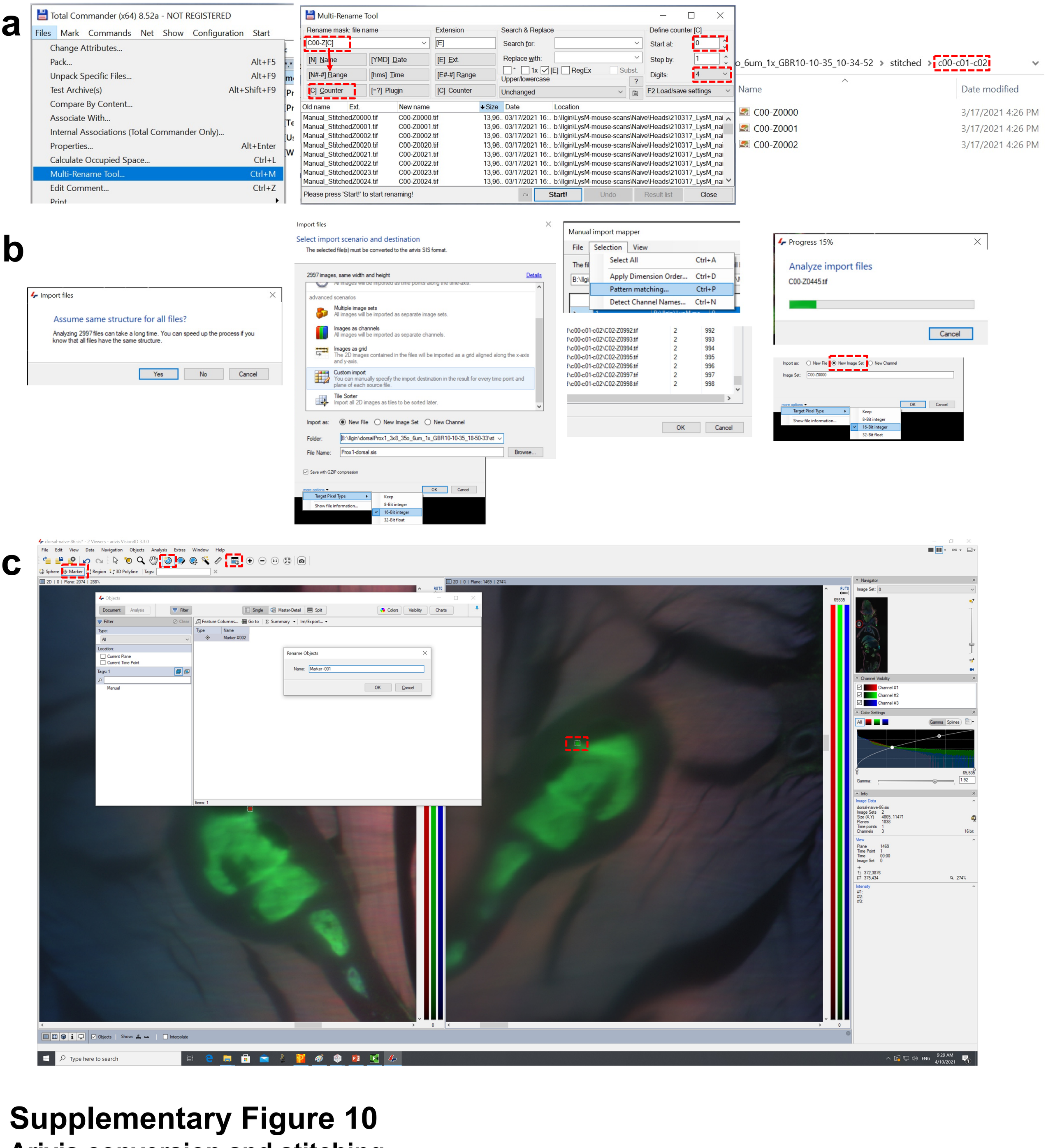




Supplementary Figure 9

2D Fiji stitching

(a) Open FIJI, go to Plugins/Stitching/deprecated/Stitch sequence of Grids of Images. **(b)** Fill in the prompter as shown and choose a optical section deep in the tissue for “start z”. **(c)** After clicking OK, stitching parameters will be calculated and resulting files will be saved in the input directory. Rename “.registered” file ending as “{zzz}.new.txt”. **(d)** Open this file and change the section number in each line to 0000. Save the changes and copy this file under a new file for the current channel. Create new folders for each channel and place this file into each folder with the changed channel information. **(e)** Load the custom macro. Click on “Run and open the txt file contacting stitching parameters. Enter the number of sections in the dataset and click “OK” to start running the macro.

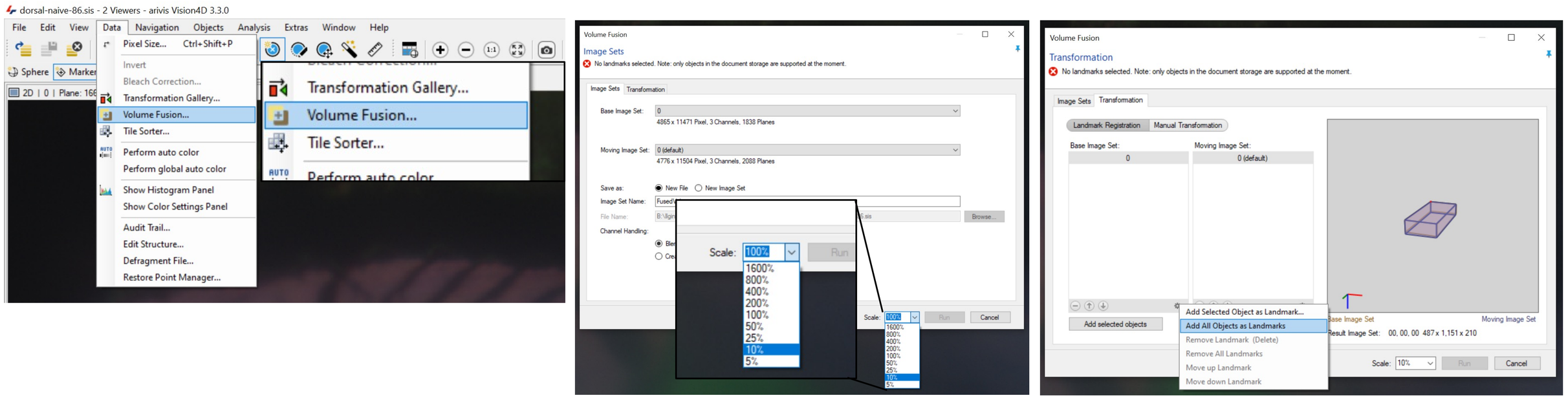


Supplementary Figure 10

Arivis conversion and stitching

(a) Rename the FIJI-stitched images (e.g. the ventral side of a whole body) with an orthodox file manager software with a multi-rename tool such as “TotalCommander“. The renamed file should include the the channel number and the z section number. Use the “Counter” function, set “Start at” as 0 and “Digits” as 4 as shown in red. Put all the renamed files into one folder and drag this folder into Arivis4D. **(b)** Click “Assume same structure for all files”. This will open another window. Choose “Custom import”, “New File” and click on more options. Choose Target Pixel Size to be 16-Bit integer and click “Ok“. This will open another window. Click on “Selection” and “Pattern matching”. Check that files will be loaded by the correct channel and section numbers. Click “Ok” to start the simultaneous conversion and import session. When the first import is finished, drag the other scan (e.g. the other side of the whole body) that needs to be fused on Arivis and follow the same steps of conversion except for one important change: choose “New Imge Set”, instead of “New File”. **(c)** When both sides are imported choose double sided view and put the different sides to each window. Using the navigation option find the same structure in both images. Choose “Place object” button, then “Marker” option, and check these landmarks through “Objects table”. Rename each landmark by right click “Rename Objects”, coresponding to the landmark number. Find at least 3 landmarks.

a



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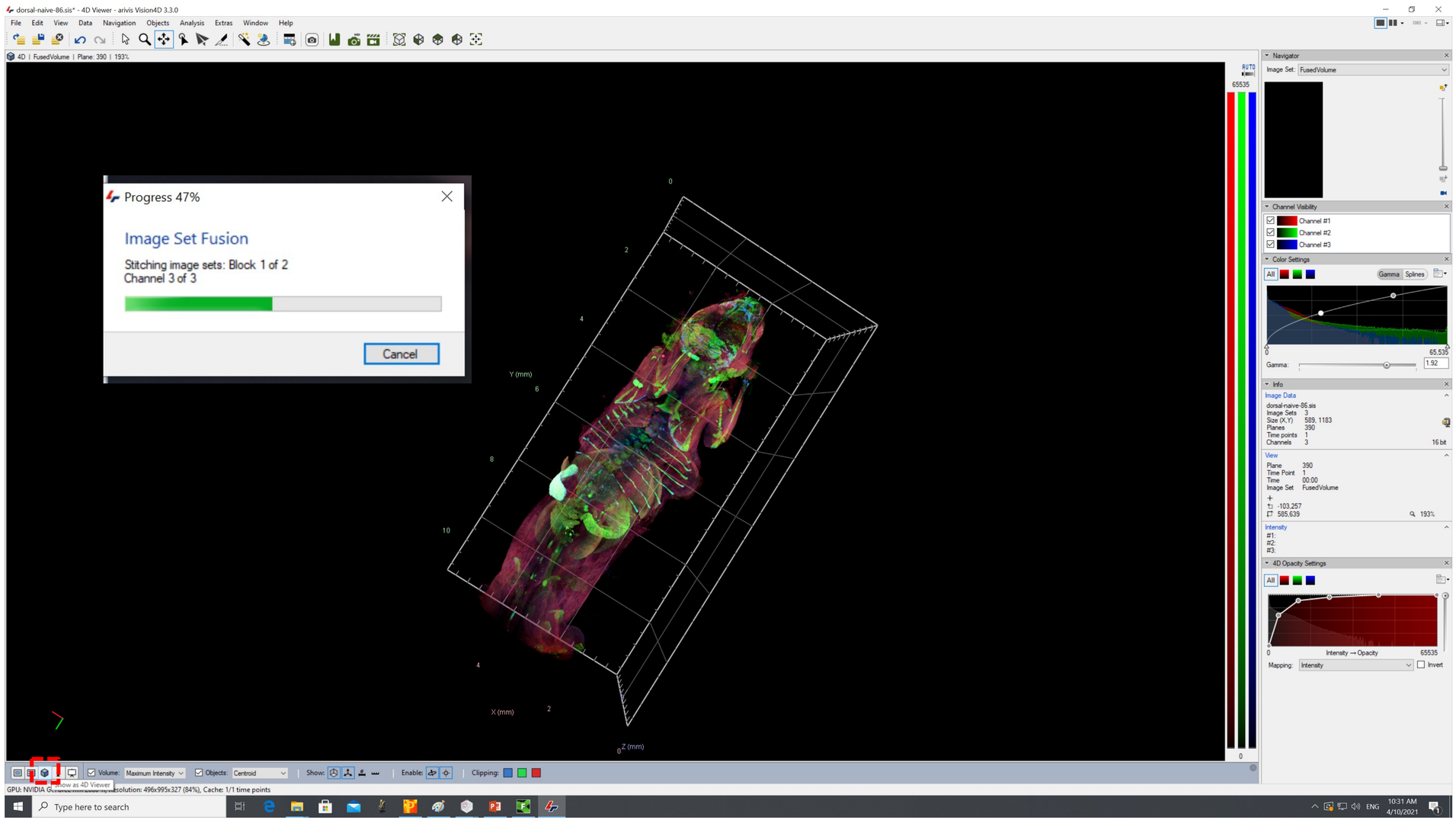

Supplementary Figure 11

Arivis stitching

(a) After three landmarks are set in each side, click on “Data” and then “Volume Fusion”. This will open a new window. Choose the base image set and the moving image set and click on New Image Set. Change the “Scale” to 10% to initially test how well the landmarks work. Click on Transformation tab to choose the landmarks for each side. Click on the wheel button at the right bottom of each image and choose “Add All Objects as Landmark”. Naming of these will help you to match landmarks from each image. Click on Run to obtain a low resolution version of your fusion. **(b)** After 10% version is ready, judge the quality of the landmarks and if sufficient start the 100% scale fusion changing the Save as option to “New File”. You can view the 3D version of your resulting fusion with the cube shaped button at the bottom left (marked with red box). After the fusion is finished, click on the “ Save“ button and export the files as a series of images using “Tiff Exporter” under File/Export. The exported files are ready for further processing.