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Whole-mouse clearing and imaging at the cellular level with vDISCO

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Homeostatic and pathological phenomena often affect multiple organs across the whole organism. Tissue clearing methods, together with recent advances in microscopy, have made holistic examinations of biological samples feasible. Here, we report the detailed protocol for nanobody(V_HH)-boosted 3D imaging of solvent-cleared organs (vDISCO), a pressure-driven, nanobody-based whole-body immunolabeling and clearing method that renders whole mice transparent in 3 weeks, consistently enhancing the signal of fluorescent proteins, stabilizing them for years. This allows the reliable detection and quantification of fluorescent signal in intact rodents enabling the analysis of an entire body at cellular resolution. Here, we show the high versatility of vDISCO applied to boost the fluorescence signal of genetically expressed reporters and clear multiple dissected organs and tissues, as well as how to image processed samples using multiple fluorescence microscopy systems. The entire protocol is accessible to laboratories with limited expertise in tissue clearing. In addition to its applications in obtaining a whole-mouse neuronal projection map, detecting single-cell metastases in whole mice and identifying previously undescribed anatomical structures, we further show the visualization 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 allows systematic and comprehensive studies of cellular phenomena and connectivity in whole bodies.

Introduction

An unbiased way to analyze an entire organism would help improve the comparative study of pathologies that affect the whole body or individual organs[1]. If we consider histology and microscopy imaging, 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 practically unfeasible, while other whole-body imaging techniques such as positron emission tomography or magnetic resonance imaging do not achieve sufficient resolution to detect morphological changes at cellular level[2–4]. The development of tissue clearing[5–10] methods that make histological samples optically transparent, deep-tissue immunolabeling techniques[11–14] and the maturation of light-sheet microscopy[15] have, over the past 15 years, allowed the adoption of a more holistic histological approach to study the structures of tissues and organs. Tissue clearing has even been applied to whole-body adult mice, paving the way to the concept of whole-body 3D histology via the detection of endogenously expressed fluorescent proteins; however, that approach relied on the removal of the skin[16–22]. Although fluorescent proteins are commonly used in microscopy, they are often not sufficiently bright to overcome the strong autofluorescence in a whole body originating from tissues such as skin, muscle and calcified bone. In addition, fluorescent proteins are quenched by the clearing reagents[16] and tend to bleach quickly during an imaging session, thus rendering multiple imaging sessions on the same sample often impossible. Cumulatively, these factors lead to signal detection of fluorescent structures being unreliable and, consequently, difficult or impossible to quantify. To address these issues, we developed a pipeline termed vDISCO (nanobody(V_HH)-boosted 3D imaging of solvent-cleared organs)[23]

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(Fig. 1) that allows easy head-to-toe imaging and the reliable analysis of an entire organism, at cellular resolution.

Development of vDISCO

In standard histology, the use of antibodies targeting the fluorescent proteins and conjugated to bright synthetic fluorophores (such as the ones from the Alexa and Atto family) represent a solution to increase the brightness and visibility of the proteins. Therefore, we reasoned that this strategy could be used to increase the signal of fluorescent proteins in an entire body.

Antibodies, however, 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 therefore decided to employ nanobodies to achieve whole-body immunolabeling (Fig. 2). In fact, nanobodies, being ten times smaller than antibodies (12–15 kDa) (refs. [24,25]), represent the smallest entities derived from immunoglobulins that are able to bind an antigen and can be conjugated to synthetic fluorophores[26].

Previously, we validated in dissected brains from CX3CR1-GFP[27] mice (expressing green fluorescent protein (GFP) in microglia) that the passive staining with nanobodies was more efficient in penetrating the tissue[23] compared with antibodies. However, we realized that passive incubation of whole mice with nanobodies alone was still not sufficient to achieve the level of extensive labeling we sought for targeting the entire body[23]. We therefore decided to exploit the cardiovascular network of the fixed animals to homogeneously inject 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[28]) (Figs. 2, 3 and 4 and Extended Data Fig. 1). Additionally, we decreased sources of autofluorescence and light-scattering at two different timepoints by adding a decolorization and a decalcification step before immunolabeling and a clearing step after immunolabeling.

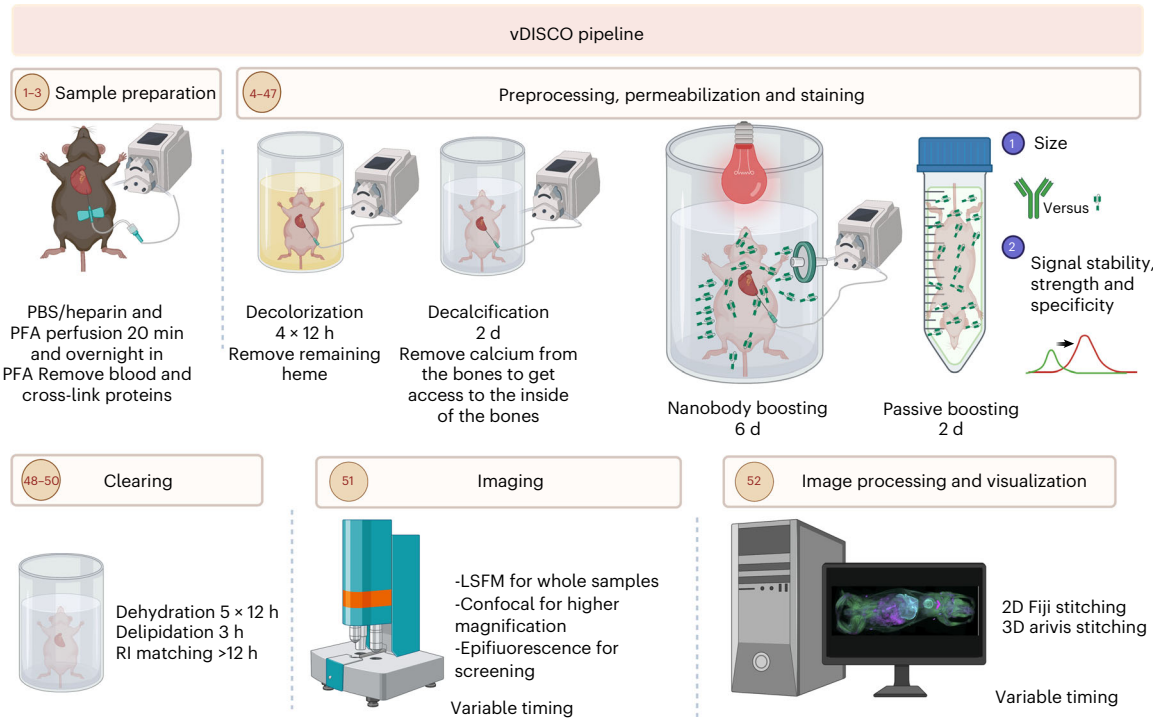


Fig. 1 | Overview of the vDISCO pipeline. Whole-body vDISCO has five main parts. After the sample is perfused with PBS and PFA (Steps 1–3), preprocessing steps such as decolorization and decalcification take ~4 d in total. Following this step, labeling and boosting take place, in which samples are incubated with nanobodies at higher temperatures for 6 d with active perfusion and 2 d in passive incubation. The use of nanobodies allows better penetration owing to their small size, and better signal-over-background ratio in cleared tissues compared with uncleared tissue owing to signal preservation and long-wavelength spectra. Additional dyes such as PI can also be incorporated here (Steps 4–47). Next, the 3DISCO clearing is performed (Steps 48–50). As a result, the cleared sample can be imaged with different microscopes: light-sheet, confocal or epifluorescence depending on the research focus (Step 51). After imaging, the data can be stitched if a tiling scan was utilized, and further analysis can take place (Step 52). The whole procedure takes ~3 weeks, and multiple samples can be processed at the same time. Figure created with BioRender.com.

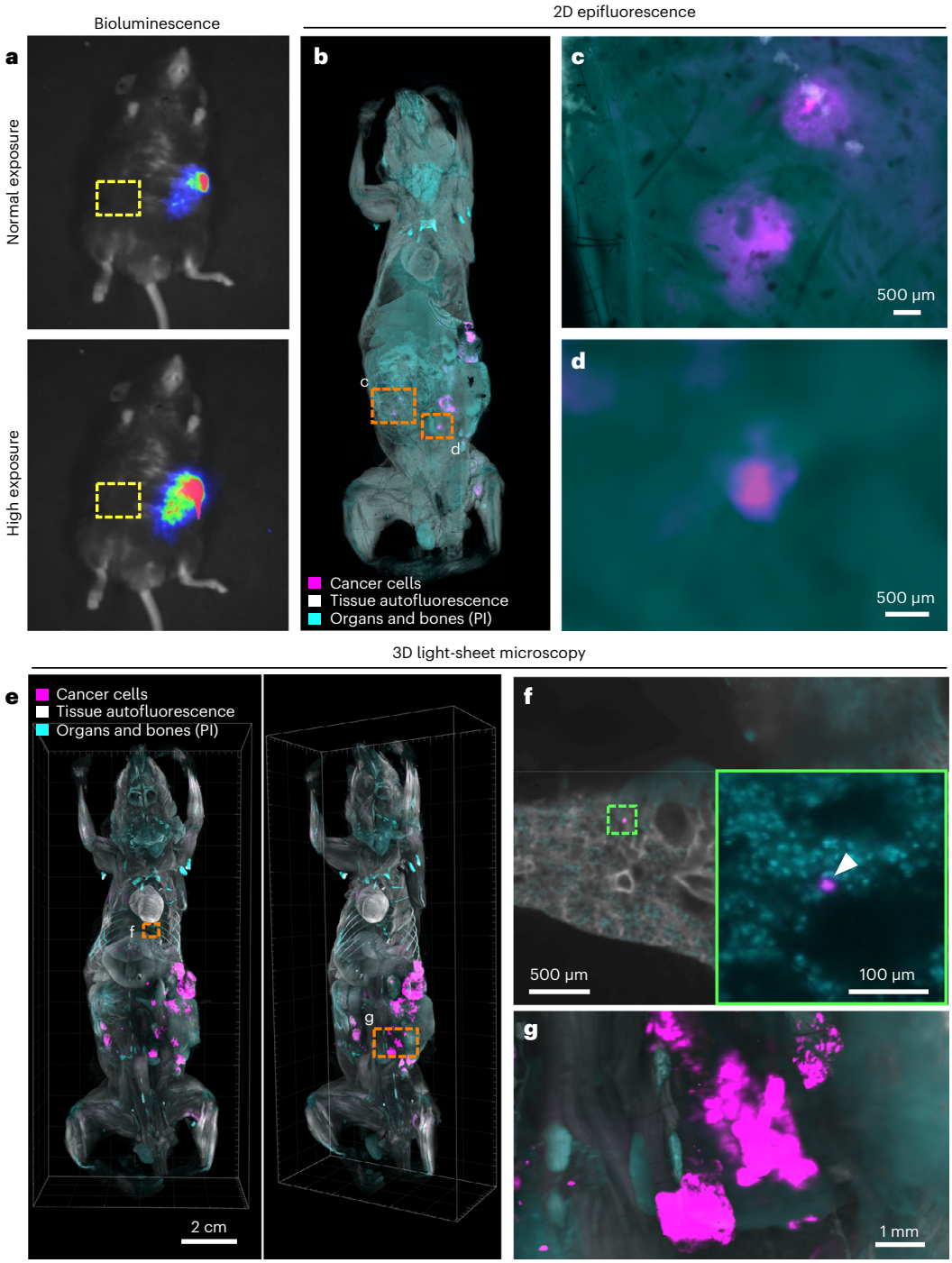
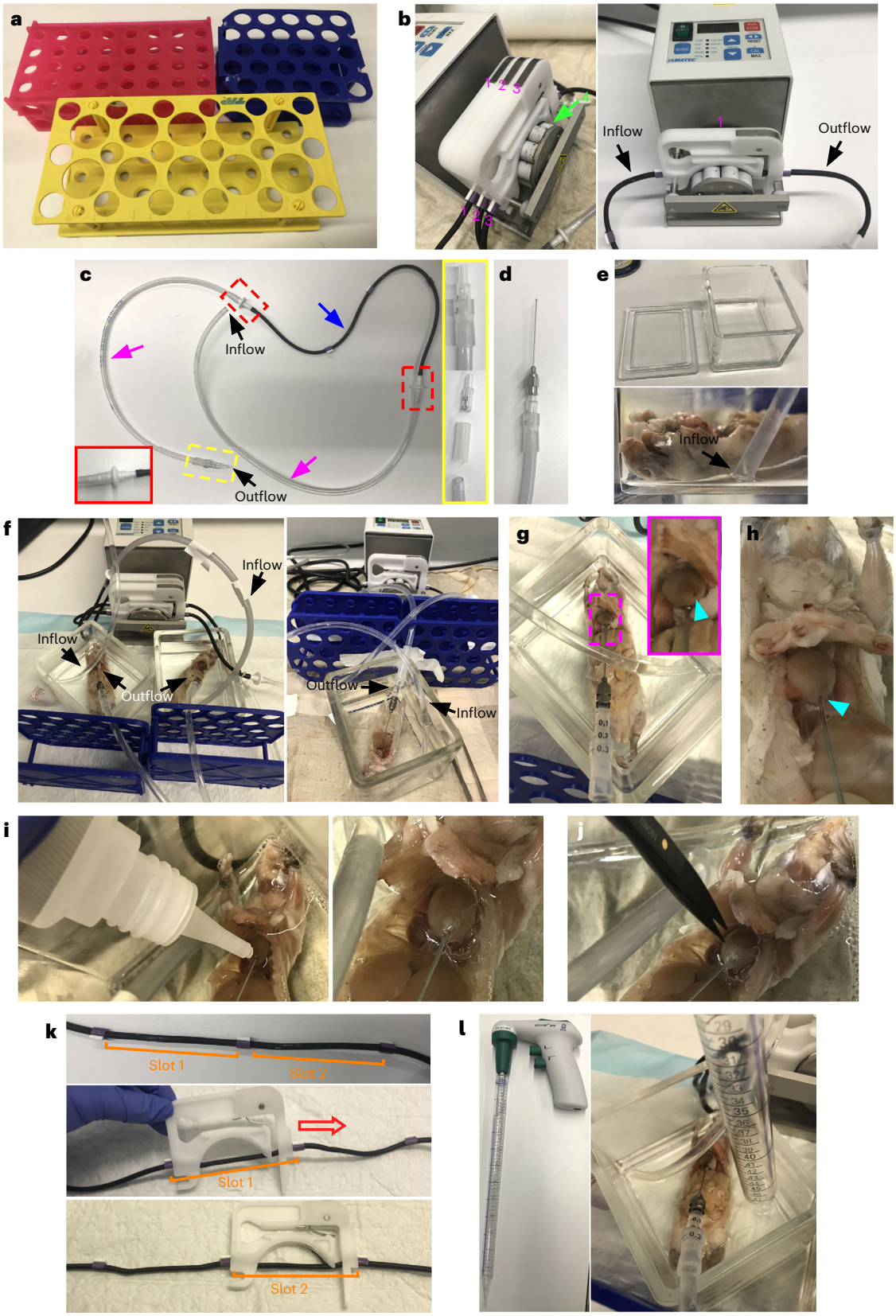


Fig. 2 | vDISCO imaging of a mouse with syngeneic pancreatic cancer. a, Normal and high exposure of bioluminescence imaging of the mouse with pancreatic cancer expressing eGFP at the end point of 38 d. **b**–**d**, 2D epifluorescence microscope imaging (**b**) of the same mouse after vDISCO labeling and clearing. The eGFP was boosted with nanobodies conjugated with Atto647N. Zoomed-in views (**c** and **d**) of the boxed regions in **b** indicate that the vDISCO pipeline provides more details of metastases (magenta) formation in the peritoneum that are not available from standard bioluminescence imaging (see **b**–**d** versus **a**). **e**, Ventral and 30° rotated views of the 3D reconstruction from light-sheet microscopy scanning of the mouse. **f**,**g**, Cellular-level-resolution light-sheet microscopy reveals individual metastases with various sizes and shapes, including a single disseminated cancer cell detected in the lungs (**f**, white arrowhead). In **b**–**g**, pancreatic cancer cells are shown in magenta, organs and bones labeled by PI are shown in cyan, and tissue autofluorescence is in white. Note that **f** was obtained by imaging the animal 2 years after vDISCO processing. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was medium.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◀ **Fig. 3 | Whole-body active-vDISCO setup. a**, Sample holders used in whole-body active-vDISCO to hold the perfusion needle. **b**, The peristaltic pump that can hold four pumping channels; therefore, it can process four bodies at the same time. Left: three channels are indicated by magenta numbers; the green arrow indicates the slot for the fourth channel. Right: only one channel shown 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), and an outflow end made from the tip of 1 ml Braun syringe (yellow boxes). **d**, Perfusion needle. **e**, Top: glass chamber used for the perfusion and the whole-body clearing steps. Bottom: position of the sucking end of the pumping tube inside the glass chamber with the tip that reaches the bottom of the chamber. **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. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.

Advantages and applications of vDISCO and comparison with other methods

In contrast to previous whole-body clearing protocols, vDISCO improves the signal-over-background ratio by synergistically combining different treatments.

First, the decalcification step reduces light scattering enabling the clearing of bones. Thus, the brain becomes optically accessible while still enclosed in the skull, with no need for dissection. vDISCO was able to confirm the presence of brain lymphatic vessels in the intact meningeal compartment[23,29,30], which would normally be damaged during the removal of the brain from the skull.

Previously we showed that, with nanobodies conjugated to far-red dyes such as Alexa647 or Atto647N, the fluorescent signal can be shifted to the far-red range. This range has the benefit of increasing the light penetration into the tissue and improving the signal-over-background ratio by reducing the autofluorescence[31]. As a result, fluorescent structures will appear in improved contrast and smaller cellular details such as neurites of neurons can be detected for image analysis in boosted samples compared with nonboosted samples. For example, neurons in scans from brains processed with vDISCO and obtained by light-sheet microscopy could be reliably traced[23] with algorithms that were implemented for higher resolution microscopy systems such as confocal[32].

The signal from the synthetic fluorophores is highly stable after clearing, and is retained for many months[23] and even years (Figs. 2 and 5). This allows the repeated imaging of the same samples for a long period of time.

Besides boosting fluorescent proteins expressed by transgenic lines, vDISCO is also compatible with virus tracing and transplanted fluorescent cells[1].

vDISCO does not require specialist and expensive equipment and could be implemented by researchers from any field. In fact, it simply consists of a series of steps where the samples are incubated or perfused with different solutions. Apart from the time needed to set up the pumping system, the actual work to change the solution takes a few minutes, allowing multiple samples to be started at the same time. Whole bodies can be processed in 2–3 weeks, while other whole-body clearing methods take 11–21 d only for the clearing step[18,21,22]. In our hands, we could run the staining and clearing of up to 18 animals at the same time by using six pumps simultaneously (three channels per each pump). This is sufficient to run an experiment with three conditions and with at least five animals per condition. It is possible to run more animals by increasing the number of the pumps. We could image 15 animals in ~2–3 months[1].

Imaging and analyzing samples in 3D is also convenient: whole organs can be imaged with the light-sheet microscope within a few hours, whole bodies within 3–14 d (depending on the imaging system), while data analysis can be concluded in 3–4 d or 1–2 weeks, again depending on the size of the data and the type of analysis. These timings are substantially shorter than the ones required in standard histology where the whole process from sectioning and reconstructing whole bodies would take several months and would 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[33–35]. Moreover, the resulting data from sectioning might present artifacts or loss of molecular and tissue architectural features due to mechanical distortions during the slicing. These problems can lead to image alignment issues and misevaluation of the data, for example, in terms of connectivity, density and topography of the objects of interest.

Samples processed with vDISCO are compatible not only with light-sheet microscopes from different manufacturers and sources (Figs. 2e–g and 5 and Extended Data Fig. 2), but also with other fluorescence imaging systems such as epifluorescence (Fig. 2b–d), confocal (Figs. 6a) and two-photon

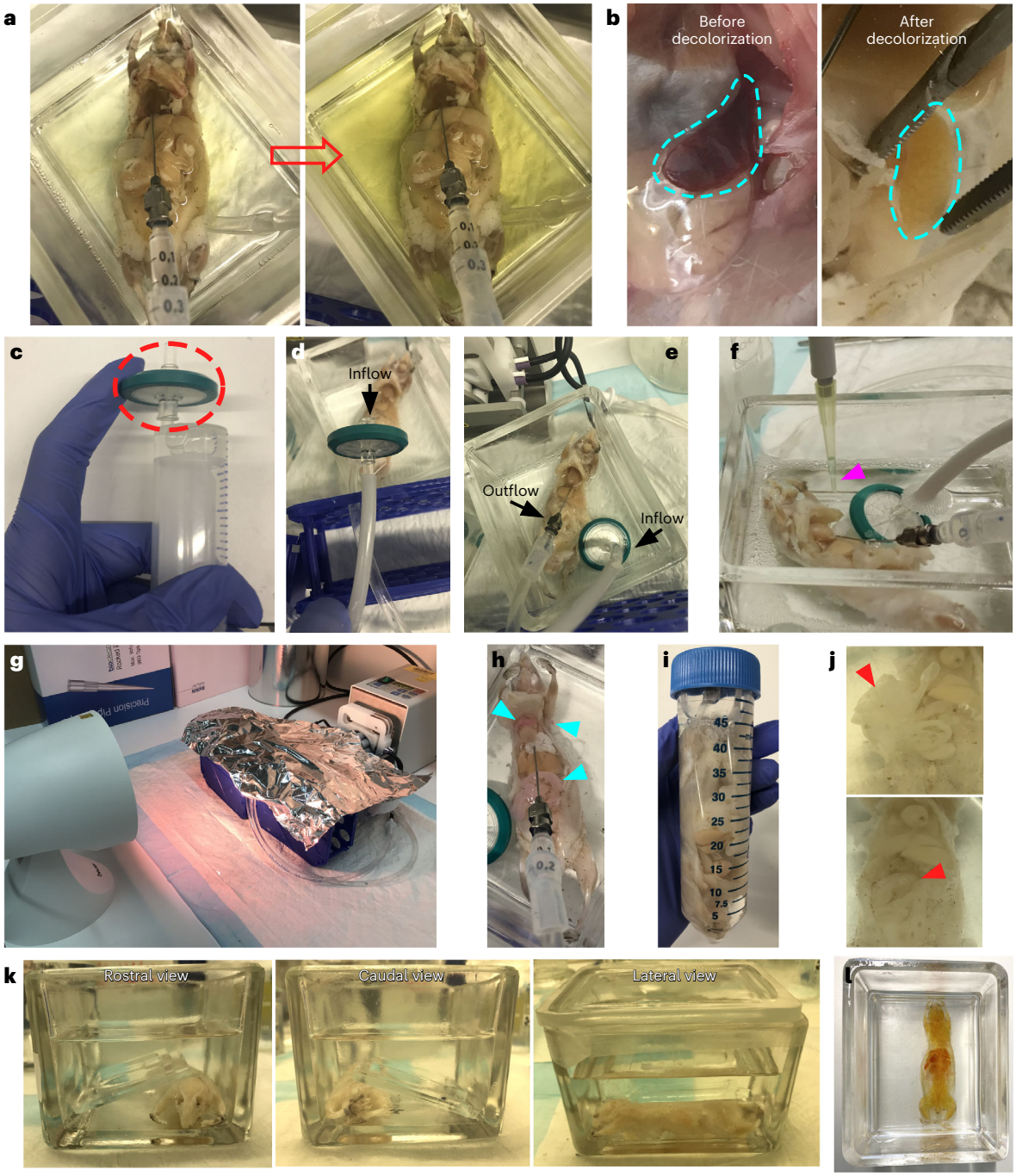

Fig. 4 | Whole-body active-vDISCO procedure. a,**b**, Pictures showing the successful elution of the heme from the body, indicated by the change of color of the decolorization solution, which turns from colorless into yellow (**a**), and the change of color of some organs such as the spleen, which turn 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 arrowhead) 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 arrowheads). **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. **l**, Dorsal view of the cleared animal inside the glass chamber. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.

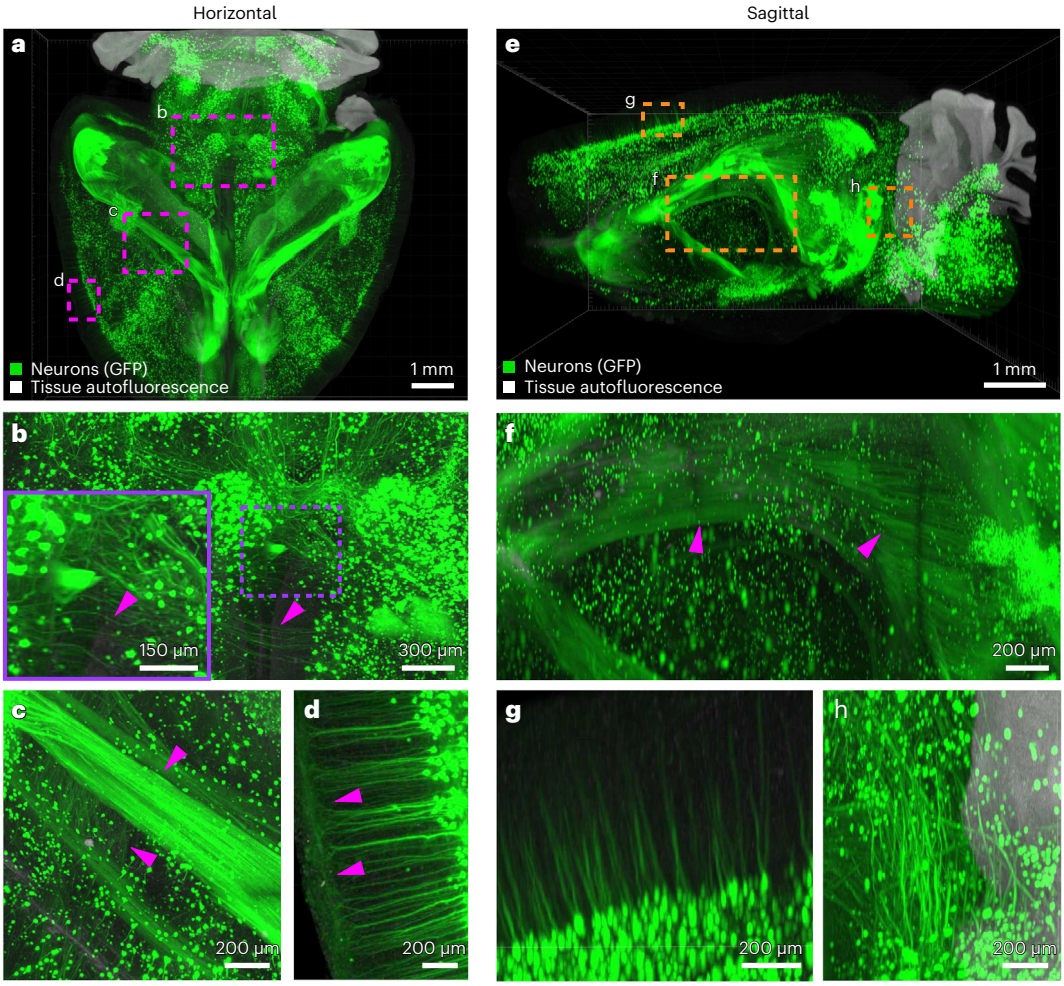

Fig. 5 | vDISCO processed Thy1-GFPM brain imaged with Z.1 light-sheet microscope. Imaris 3D reconstruction of a Thy1-GFPM mouse brain imaged by the Lightsheet Z.1 microscope 2 years after vDISCO processing. **a**–**h**, The brain was imaged horizontally and it is shown in horizontal view (**a**–**d**) and, after rotation of the 3D reconstruction volume, in sagittal view (**e**–**h**). **b**–**d**, Zoom-in images of the areas indicated by the boxed regions in **a**, showing commissural axons (**b**, arrowheads) 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**, arrowheads). **f**–**h**, Zoom-in of the areas indicated by the boxed regions in **e**, showing neuronal details after rotating the brain to sagittal view: bundles of corpus callosum axons (**f**, arrowheads), pyramidal cortical neurons (**g**) and commissural axons (**h**) are visible with continuity even in sagittal view because of the isotropic resolution of the microscope. Similar results were achieved at least in two different samples. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.

microscopes (unpublished observations; R.C.). This versatility is useful because each imaging system has specific advantages such as higher resolution for confocal or speed for epifluorescence.

With the above-mentioned features, vDISCO can pave the way for a new histological approach capable of addressing the complexity of entire biological systems. This prevents potential bias when examining only a particular region of interest and the risk of overlooking important events in other regions. For example, vDISCO was used to reconstruct the neuronal projection map[23] of a Thy1-GFPM mouse where a subset of neurons expresses GFP[36]. This achievement led to the discovery of peripheral neuronal degeneration after traumatic brain injury[23]. vDISCO was also 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 mice with cancer[1].

With the clearing of entire heads, vDISCO was applied to comprehensively study the infiltration of peripheral monocytes/macrophages into the brain after stroke. This contributed to the elucidation and discovery of new routes called short skull–meninges connections that peripheral immune cells take to invade the cerebral tissue[23]. vDISCO can further clear whole mice with intact skin (Extended

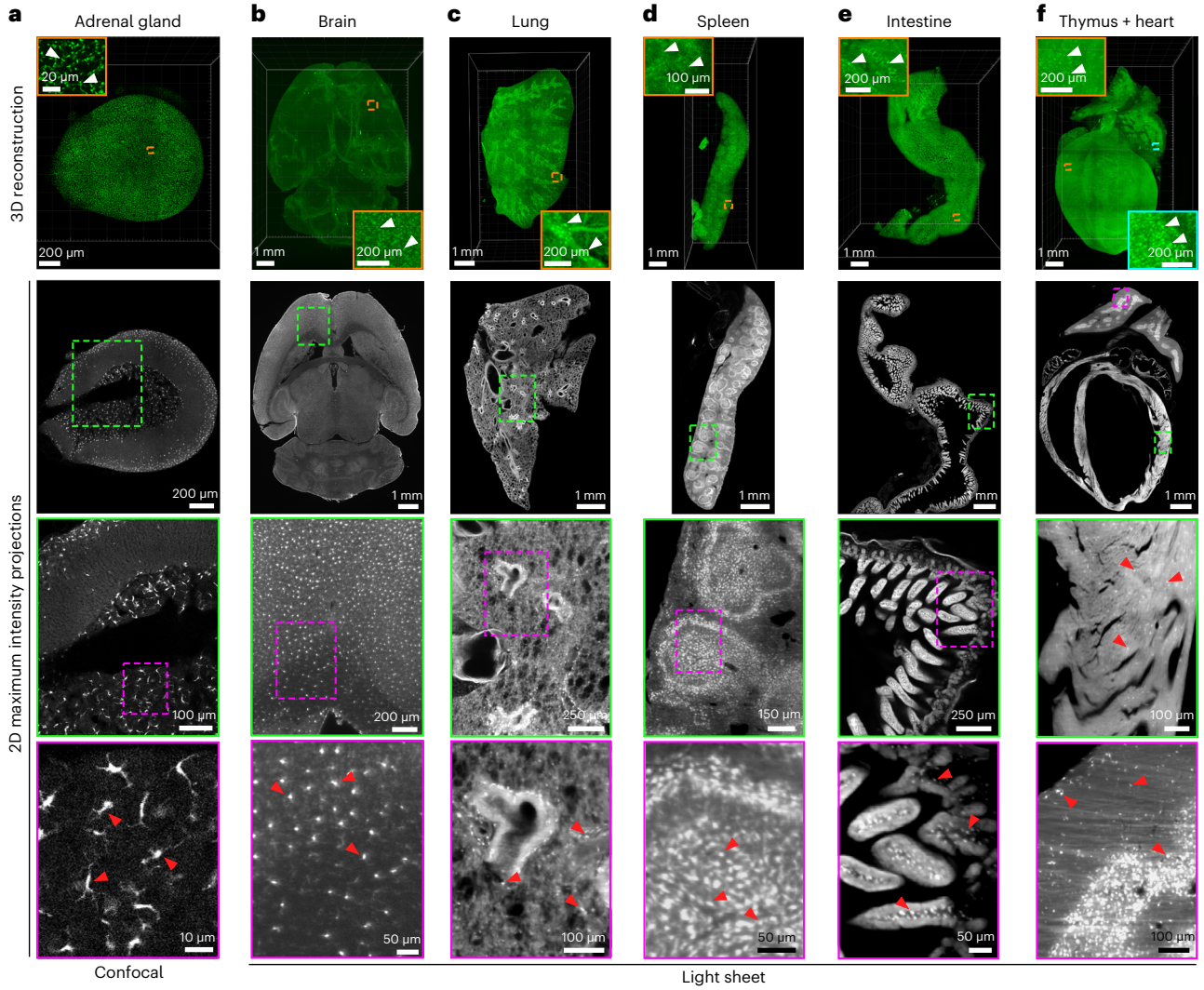

Fig. 6 | Passive-vDISCO on dissected organs imaged with confocal and light-sheet microscopy. a–f, 3D reconstructions and 2D maximum intensity projections of an adrenal gland (**a**), dissected brain (**b**), lung lobe (**c**), spleen (**d**), intestine segment (**e**) and thymus + heart (**f**) from the CX3CR1-GFP mouse line after passive-vDISCO. Zoom-in images from the dashed regions show individual CX3CR1 GFP+ immune cells (red arrowheads). To remove residual blood, the organs in **c** and **d** were initially decolorized for 2 d in the decolorization solution (25% dilution of the stock), while the ones in **e** and **f** were decolorized for 1 d in the decolorization solution (20% dilution of the stock). Similar results were observed in two independent animals. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.

Data Fig. 2), while all other previous methods required the removal of this organ[16–22] enabling the visualization of immune cells and sensory nerves in the skin and their connectivity with the spinal cord[23].

vDISCO was further used to boost the fluorescence signal in intact organs of large animal models; for instance, it was used to quantify and evaluate the distribution of β-cell islets in the pancreas of transgenic pigs expressing GFP[37].

Limitations

The use of organic solvents in the clearing step of vDISCO can decrease the retention of the proteins in the tissue, compared with hydrogel embedding methods[38]. As a result, low-expressed proteins can be difficult to detect. As the decolorization and clearing also delipidate the tissues, the retention of lipids and lipid-associated proteins might be affected by the approach[16,39]. For the same reason, lipophilic dyes such as DiI and myelin staining, previously shown as incompatible with organic-solvent-based methods[5], might not be detectable after vDISCO. Similarly, the success of vDISCO on

transgenic lines expressing fluorescent proteins related to lipid-associated proteins should be carefully evaluated, although a proper and extensive fixation of the tissue might help. In addition, samples deprived of lipids might not be compatible with electron microscopy[10].

With nanobodies binding to fluorescent proteins, vDISCO can theoretically boost up to 21 types of fluorescent protein, including mCherry and Venus. Validated fluorophores and fluorescent proteins are presented in Table 1. vDISCO is in principle compatible with nanobodies targeting endogenously expressed proteins as well[23]. However, the use of vDISCO as a primary staining approach is limited by the lack of nanobodies developed for histology. Nanobodies were in fact mostly optimized for in vivo applications such as therapeutic agents or as labels for intracellular markers in living cells[40]. The potential future development of nanobodies suitable for deep tissue immunolabeling of fixed thick specimens, as well as that of near-infrared fluorophores conjugable to the nanobodies, may expand the vDISCO applications and further the possible imaging depth[31].

A dedicated microscope is also required to scan the samples that are much larger than traditional slides. Typical organic-solvent-based clearing methods in fact shrink the samples up to 65% of their original volume[16,23], resulting in cleared mice (10 cm in length after shrinkage, 13–15 cm if the limbs are stretched) that could be imaged from head to toe but were still substantially bigger than the travel range of a light-sheet microscope developed for entire organs, in which case imaging of a whole animal could not be concluded in one session; rather, the sample was continuously displaced or flipped in order to image all the body parts. The resulting 20–24 individual scans acquired over 2–3 weeks were then stitched together[16,23]. The recent availability of light-sheet microscopes with bigger imaging chambers and larger sample holder stages[1,37] has importantly reduced the time and the amount of work spent during the data acquisition. Currently, in these systems, the image acquisition only takes 3–4 d 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. We note that the rate of shrinkage must be carefully evaluated in different organs and tissues.

Lastly, the amount of raw data generated can be difficult to handle: the scans of a single animal can occupy 2–3 terabytes[23], to be analyzed with at least 256–512 gigabytes of computer random-access memory (RAM), which is not always available. Moreover, data sizes of hundreds or thousands of gigabytes are too complex or not feasible to be analyzed by a human operator. Algorithms that can automatically analyze the collected information are discussed later in the 'Data analysis and processing' section.

Experimental design: overview of the vDISCO pipeline

The vDISCO pipeline consists of sequential steps: sample preparation (Steps 1–3), decolorization, decalcification, permeabilization and staining (Steps 4–47), and tissue clearing (Steps 48–50). Following these steps, samples are ready to be imaged (Step 51) and be subjected to subsequent image processing, visualization and analysis pipelines (Step 52) (Fig. 1). If performed by a relatively experienced user, after validating the protocol in a pilot experiment with one animal, vDISCO would show a success rate of nine out of ten samples.

Choice of the sample and sample preparation

vDISCO is a robust method that has been extensively tested and reproduced in different experiments (Figs. 2, 5–9 and Extended Data Figs. 2 and 3). We recommend starting with samples bearing GFP, YFP, mCherry or RFP. For transgenic animals, genotyping should be performed to confirm the expression of the fluorescent protein. In addition, 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).

A simpler version of vDISCO called 'passive-vDISCO'[23] (Tables 1–3, Figs. 6, 7 and 9, Extended Data Fig. 3 and 'Procedure' section) that has optional decolorization and decalcification steps and requires passive incubation can be applied on dissected organs and small samples such as embryos, half (Fig. 7 and Extended Data Fig. 3) or whole brains with spinal cord (Extended Data Fig. 4), lungs, gut, adrenal glands from mice (Fig. 6) and whole small pig organs such as pancreas[37]. Passive-vDISCO can also be used in sections to collect experimental data or quickly test and assess the performance of new nanobodies (Box 1). Both slices and dissected organs can be imaged with a broad variety of microscope systems, including confocal, epifluorescence and light-sheet microscopes (Figs. 6–9 and Extended Data Fig. 3). The decision to choose between whole-body vDISCO or

Table 1 | Nanobodies tested with vDISCO

Nanobody	Known target	Tested compatibility[a]	Company	Cat. no.	Lot/batch	Suggested concentration[b]	Protocol used	Tested samples	Performance	Note
GFP-Booster Atto647N (AB_2629215)	Anti-GFP	YFP, eYFP, eGFP	Chromotek	gba647n-100	60920001SAT2	Stock = 0.5–1 mg/ml Mouse brain: 1:500–1:700 (1.1–1.8 µg/ml) Whole mouse: 1:7,000 (0.08–0.14 µg/ml)	Standard passive and active vDISCO	Mouse organs and mouse whole body	Very bright, good penetration	14 d incubation for a whole brain
GFP-Booster Atto647N (AB_2629215)	Anti-GFP	YFP, eYFP, eGFP	Chromotek	gba647n-100	70213001SAT2	Stock = 0.5–1 mg/ml Mouse brain: 1:500–1:700 (1.1–1.8 µg/ml) Whole mouse: 1:7,000 (0.08–0.14 µg/ml)	Standard passive and active vDISCO	Mouse organs and mouse whole body	Very bright, good penetration	14 d incubation for a whole brain
GFP-Booster Atto647N (AB_2629215)	Anti-GFP	YFP, eYFP, eGFP	Chromotek	gba647n-100	71017011AT2	Stock = 0.5–1 mg/ml Mouse brain: 1:500–1:700 (1.1–1.8 µg/ml) Whole mouse: 1:7,000 (0.08–0.14 µg/ml)	Mild-vDISCO for passive staining	Mouse brain	Good signal and penetration	No signal using standard passive-vDISCO
GFP-Booster Atto647N (AB_2629215)	Anti-GFP	–	Chromotek	gba647n-100	80515001AT2	–		Mouse brain	Very weak signal	Withdrawn from the market by the company
GFP-Booster Atto647N (AB_2629215)	Anti-GFP	YFP, eYFP, eGFP	Chromotek	gba647n-100	90107001SAT2	Stock = 0.5–1 mg/ml Mouse brain: 1:500–1:700 (1.1–1.8 µg/ml) Whole mouse: 1:7,000 (0.08–0.14 µg/ml)	Mild-vDISCO for passive staining Standard vDISCO for active staining	Mouse organs and mouse whole body	In passive-vDISCO signal not so bright but visible, good penetration. In active-vDISCO good performance	14 d incubation for a whole brain
GFP-Booster 2AlexaFluor647 (AB_2827575)	Anti-GFP	YFP, eYFP, eGFP	Chromotek	gb2AF647-50	02TEC77	Stock = 0.5–1 mg/ml Mouse brain: 1:400–1:800 (0.6–2.5 µg/ml)	Mild-vDISCO for passive staining	Mouse brain	Bright, good penetration	14 d incubation for a whole brain
GFP-Booster 2AlexaFluor647 (AB_2827575)	Anti-GFP	YFP, eYFP, eGFP	Chromotek	gb2AF647-50	90212037AX2	Stock = 0.5–1 mg/ml Mouse brain: 1:400–1:800 (0.6–2.5 µg/ml	Mild-vDISCO for passive staining	Mouse brain	Bright, good penetration	14 d incubation for a whole brain
GFP-Booster 2AlexaFluor647 (AB_2827575)	Anti-GFP	–	Chromotek	gb2AF647-50	90524037AX2	–	Standard passive-vDISCO	1 mm mouse brain slices	Very weak signal	–

Table continued

Table 1 (continued)

Nanobody	Known target	Tested compatibility[a]	Company	Cat. no.	Lot/batch	Suggested concentration[b]	Protocol used	Tested samples	Performance	Note
GFP-Booster Atto488 (AB_2631386)	Anti-GFP	YFP, eYFP, eGFP	Chromotek	gba488-100	90305001AT1	Stock = 0.5–1 mg/ml Mouse brain: 1:500–1:700 (1.1–1.8 µg/ml)	-	Mouse brain	Very weak signal	-
GFP-Booster Atto594 (AB_2631387)	Anti-GFP	YFP, eYFP, eGFP	Chromotek	gba594-100	81212001AT3	Stock = 0.5–1 mg/ml Mouse brain: 1:500–1:700 (1.1–1.8 µg/ml)	Mild-vDISCO for passive staining	Mouse brain	Bright, good penetration	-
RFP-Booster Atto647N (AB_2631391)	Anti-RFP	mCherry	Chromotek	rba647n-100	81106002SAT2	Stock = 0.5–1 mg/ml Mouse brain: 1:500–1:700 (1.1–1.8 µg/ml) Whole mouse: 1:7,000 (0.08–0.14 µg/ml)	Standard active-vDISCO	Mouse whole body[1]	Bright, good penetration	-
RFP-Booster Atto594 (AB_2631390)	Anti-RFP	mCherry	Chromotek	rba594-100	71115002AT3	Stock = 0.5–1 mg/ml Mouse brain: 1:500–1:700 (1.1–1.8 µg/ml) Whole mouse: 1:7,000 (0.08–0.14 µg/ml)	Standard active-vDISCO	Mouse whole body[1,23]	Bright, good penetration	-
Fluotag-X4 anti-GFP AF647 (AB_2905517)	Anti-GFP	eGFP	NanoTag Biotechnologies	N0304-AF647	03190302	Stock = 1.25 µM Mouse brain: 1:300–1:500	Standard passive-vDISCO	Mouse brain and 50 µm slices	Very bright, penetration ok for half brain of mice	Increase the incubation time more than 30% with respect to the Chromotek nanobodies
Fluotag-X4 anti-GFP Atto647N (AB_2905516)	Anti-GFP	eGFP	NanoTag Biotechnologies	N0304-At647N	03190101	Stock = 1.25 µM Mouse brain: 1:300–1:500	Standard passive-vDISCO	1 mm mouse brain slices	Bright signal, very poor penetration	-
Fluotag-Q anti-GFP AF647 (AB_2905515)	Anti-GFP	eGFP	NanoTag Biotechnologies	N0301-AF647	03190101	Stock = 5 µM Mouse brain: 1:400–1:800	Standard passive-vDISCO	Mouse brain 50 µm slices	Very bright, good penetration	Increase the incubation time more than 30% with respect to the Chromotek nanobodies. Good results for microglia and pericytes in the brain
Fluotag-X4 anti-RFP AF565 (AB_2905518)	Anti-RFP	dsRed	NanoTag Biotechnologies	N0404-AF565	-	Stock = 1.25 µM Mouse half brain: 1:300–1:500	Standard passive-vDISCO	Mouse brain	Bright	8 d incubation for half brain

Table continued

Table 1 (continued)

Nanobody	Known target	Tested compatibility[a]	Company	Cat. no.	Lot/batch	Suggested concentration[b]	Protocol used	Tested samples	Performance	Note
Fluotag-X4 anti-RFP AF647 (AB_2905526)	Anti-RFP	tdTomato, mcherry	NanoTag Biotechnologies	N0404-AF647		Stock = 1.25 μM 1:500	Standard passive-vDISCO	Mouse half brain and 50 μm slices	Bright, moderate penetration	Increase the incubation time more than 30% with respect to the Chromotek nanobodies
Fluotag-X2 anti-mScarlet-i AF647 (AB_2905527)	Anti-mScarlet-i	tdTomato, mcherry	NanoTag Biotechnologies	N1302-AF565		Stock = 1.25 μM 1:350–1:500	Standard passive-vDISCO	Mouse half brain	Moderate penetration	Increase the incubation time more than 30% with respect to the Chromotek nanobodies
Fluotag-Q anti-RFP AF647 (AB_2905529)	Anti-RFP	tdTomato, mcherry	NanoTag Biotechnologies	N0401-AF647		Stock = 1.25 μM 1:350–1:500	Standard passive-vDISCO	Mouse half brain and 50 μm slices	Good penetration	Increase the incubation time more than 30% with respect to the Chromotek nanobodies
Custom-made anti-GFP AlexaFluo647; AB_2905530	Anti-GFP	eGFP	-	-	-	Stock = 0.46 mg/ml Mouse brain: 1:300–1:400 (1.38–1.5 μg/ml)	Standard passive-vDISCO	Mouse brain	Bright, good penetration	Increase the incubation time more than 20% with respect to the Chromotek nanobodies. Good results for staining neuronal lines

[a]According to the companies´ website: Chromotek anti-GFP nanobodies should be also compatible with CFP, wtGFP, GFP S65T, AcGFP, TagGFP, tagGFP2, mClover (Clover A206K), sfGFP, pHluorin, Venus and Citrine; Chromotek anti-RFP nanobodies should be also compatible with mRFP, mRFPruby and mPlum; Nanotag anti-GFP nanobodies should be also compatible with mEGFP, superfolder GFP and most common CFP and YFP variants; the clone 2B12 of the Nanotag anti-RFP/mScarlet-i nanobody should be also compatible with mOrange2, dsRed1, dsRed2, mRFP and mCherry. [b]Concentrations should be adjusted on the basis of sample size, expression of the fluorescent proteins and preliminary tests.

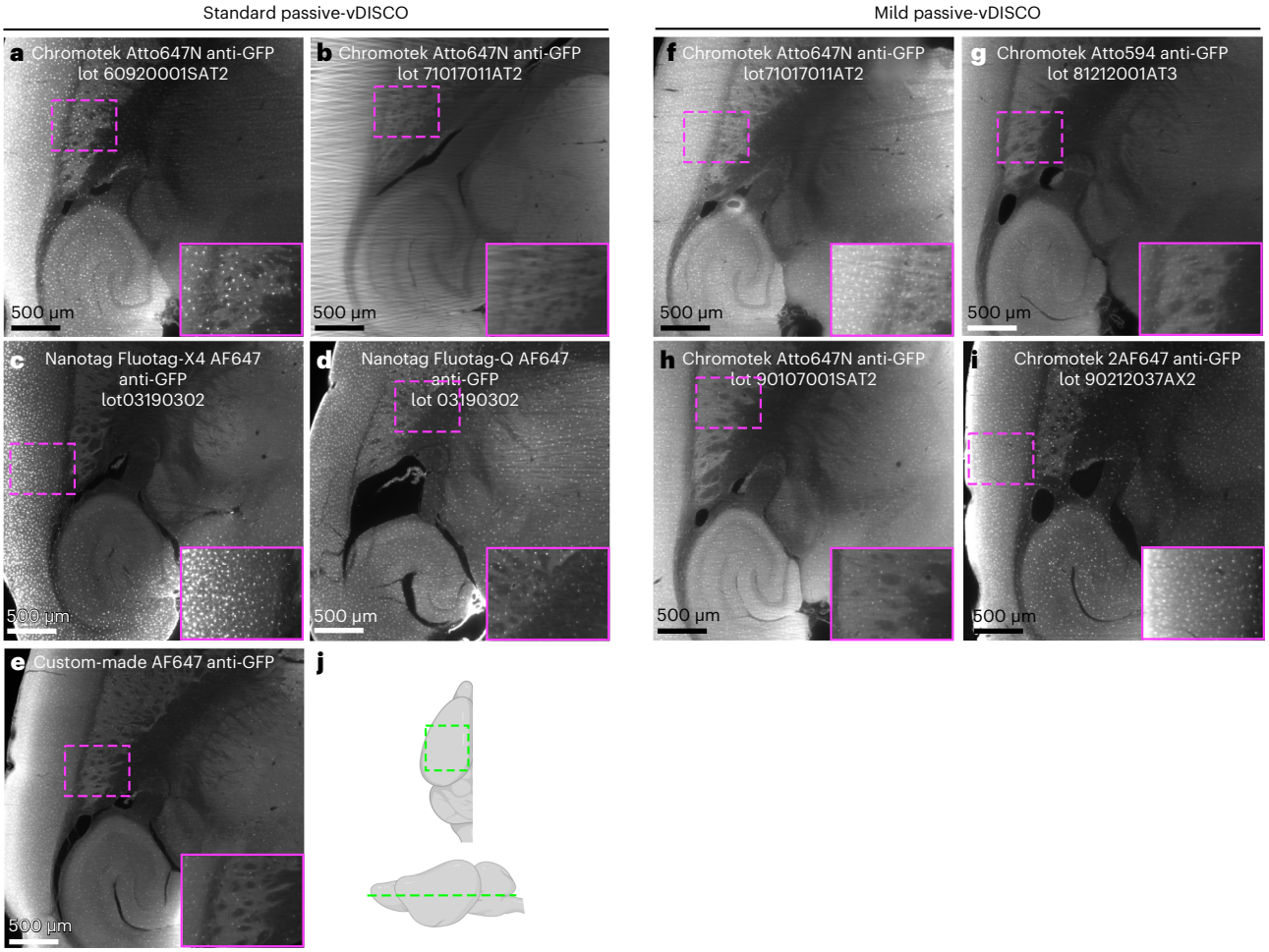

Fig. 7 | Performances of different kinds of nanobodies in passive-vDISCO. a–i, Light-sheet microscopy images of half mouse brains from CX3CR1-GFP line showing the performance of different batches of nanobodies from different sources and companies using standard passive-vDISCO (**a–e**) and mild passive-vDISCO (**f–i**). The nanobody that 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 two independent experiments for each kind of nanobody. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines in Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.

Box 1 | Nanobody validation in sections

Procedure

1 Cut a post-fixed organ of interest (e.g., brain) in 400–1,000 µm sections and place them into the wells of a 24-multiwell plate filled with 1× PBS, 0.5–1 ml each well. Cutting can be performed, for example, with a vibratome or a brain matrix.
2 Before staining, image the sections with a fluorescence microscope (e.g., confocal or epifluorescence) to verify that the sample bears fluorescence signal.
3 Incubate the sections completely immersed in the permeabilization solution for 3 h at 37 °C in the dark and with gentle shaking.
4 To test whether the nanobody is more suitable for passive-vDISCO or mild-vDISCO, allocate some sections to be incubated with the passive-vDISCO staining solution and some sections to be incubated with the mild-vDISCO staining solution. Exchange the permeabilization solution with the staining solutions (500 µl to 1 ml final volume each well) containing the nanobody and incubate overnight to 1 d at 37 °C in the dark and with gentle shaking. For the nanobody concentration, follow the information in Table 1 or the manufacturer datasheet.
▲ **CRITICAL STEP** Keep some sections that are not incubated with the nanobody as negative controls. Seal the 24-multiwell plate with tape or parafilm to prevent evaporation of the solutions.
5 Wash with the washing solution at RT three to four times for 10–20 min in the dark with gentle shaking.
6 Wash with 1× PBS at RT three to four times for 10–20 min in the dark with gentle shaking.
7 Image the stained slices with a confocal or epifluorescence microscope.

passive-vDISCO should be made before starting the whole pipeline, since passive-vDISCO requires the removal of the specimens of interest from the body.

Decolorization and decalcification

In whole-body vDISCO, the subsequent step after fixation is the perfusion with a decolorization solution, which has the purpose of removing the pigment heme contained in the blood. In the standard procedures to prepare the samples, perfusing with PBS before fixation does not completely wash the blood from organs rich of blood such as spleen and bone marrow; consequently, they will still appear dark red[16]. Heme in the blood is known to absorb light in the visible spectrum[31,41,42] and cause autofluorescence[31,43].

The decolorization solution is made by diluting ¼ CUBIC reagent #1 (CUBIC#1) solution[8,18] in PBS. The resulting solution contains Quadrol, which is the chemical with the strongest decolorization activity in CUBIC#1. In fact, Quadrol belongs to the chemical family of aminoalcohols, compounds that have previously been shown to efficiently elute heme from the blood[8,17–19,44]. Since CUBIC#1 is a very viscous reagent, in vDISCO whole-body perfusion system, only a less viscous 20–30% dilution of the reagent can be pumped. The dilution that retains a good decolorization ability is 25%, but this value should still be adjusted on the basis of the pumping system and the amount of blood present in the body. Other strategies for decolorization 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[45–47].

In passive-vDISCO, the decolorization step is performed by passively incubating the samples with the decolorization solution. Since in this circumstance the reagents can only react with the sample via diffusion (substantially slower than via the active transport system), an incubation at 37 °C would help speed up the process (Table 2 and 'Procedure' section).

The decolorization step is followed by the decalcification of the bones. Bones, owing to their calcified nature and poor content of lipids, can interfere with imaging and impede light penetration[16,48]. We decided to exploit the calcium-chelating properties of ethylenediaminetetraacetic acid (EDTA), which has been successfully used in other bone-clearing protocols[21,22,49], to remove Ca^{2+} ion from the bones[21,22,49]. The vDISCO decalcification solution consists of 10% (wt/vol) EDTA dissolved in PBS and is pumped into the body of the animal via intracardial perfusion at room temperature for 2 d. We do not recommend acids including nitric acid or hydrochloric acid previously reported as decalcifying reagents[50], because they can disrupt the antigens in the tissue.

Although the decolorization and decalcification steps can be skipped when using passive-vDISCO, organs rich of blood (e.g. spleen) or dissected bones would still benefit by the two treatments after the sample preparation and before staining (Table 2).

Staining and choice of the dyes/nanobodies

In whole-body vDISCO, the staining step can start after the animal is decalcified and washed 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, which also has the aim to loosen the extracellular matrix, 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 (Fig. 4c–h), while in passive-vDISCO both parts are performed with passive incubation at 37 °C. The warm temperature is fundamental to increase the penetration of the dyes[11,51]. In the permeabilization part, the samples are perfused/incubated for 1 d with a permeabilization solution containing serum, Triton X-100, methyl-β-cyclodextrin, *N*-acetyl-L-hydroxyproline and sodium azide in PBS. Methyl-β-cyclodextrin has the purpose to destabilize the tissue collagen[10], while *N*-acetyl-L-hydroxyproline to extract the cholesterol[10]. Then the samples are stained by perfusing/incubating with this same solution containing the dye: 6 d 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 d for 1 mm sections, 14 d for whole mouse brains, Table 2). For some batches of nanobody that show poor stability in this staining solution (Table 1), dyes should be diluted in another solution with 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). However, the absence of these two permeabilization reagents during the staining step can result in less dye penetration.

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.

Table 2 | Tested experimental timings and solution volumes for passive-vDISCO

Sample	Decolorization time (optional, depending on the blood content)	Decalcification time	Final incubation volume	Permeabilization (37 °C) time	Staining (37 °C) time[a]	Washing (RT) time	50%–70%–80%–100% THF dilutions (RT) time	Final 100% THF (RT) time	DCM (RT) time	BABB (RT) time
500-µm-thick mouse brain slices (for nanobody validation)	–	–	500–700 µl	20 min	3 h	10 min each wash	20 min	30 min	10 min	At least 20 min
1-mm-thick mouse brain slices	–	–	1 ml	4–5 h	2–3 d	20–30 min each wash	45 min	45 min + 3 h	15 min	At least 2 h
Mouse small organs such as lymph nodes or adrenal glands	–	–	1.5 ml	12 h	4 d	20–30 min each wash	1 h	1 h + 2h	30 min	At least 3 h
Half mouse brain	–	–	3–4 ml	0.5–1 d	7–9 d	30–60 min each wash	1 h	1 h + overnight	30–60 min	At least 6 h
Whole mouse brain	–	–	4–4.5 ml	1–2 d	13–15 d	1–2 h each wash	2 h	2 h + overnight	1–2 h	At least 12 h
Mouse brain + spinal cord	–	–	10–13 ml	1–2 d	13–15 d	1–2 h each wash	2 h	2 h + overnight	1–2 h	At least 12 h
Mouse spinal cord	–	–	3–4 ml	5–7 h	4–5 d	20–30 min each wash	45 min	45 min + 8 h (or overnight)	30–45 min	At least 4 h
Mouse lung lobes	If blood content is high: 2 d in decol. solution (20% (vol/vol) of stock in PBS)	–	1 ml	12 h to 1 d	3–4 d	45 min to 1 h each wash	2 h	2 h + 8 h (or overnight)	1–2 h	At least 5 h
Mouse gut tracts	If blood content is high: 1 d in decol. solution (20% (vol/vol) of stock in PBS)	–	2–2.5 ml	1 d	5–6 d	45 min to 1 h each wash	1.5 h	1.5 h + 6 h (or overnight)	1 h	At least 4 h
Mouse whole spleen	If blood content is high: 2 d in decol. solution (25% (vol/vol) of stock in PBS)	–	1 ml	1 d	7–8 d	1–1.5 h each wash	2 h	2 h + overnight	1–2 h	At least 12 h
Mouse whole thymus + whole heart	If blood content is high: 1 d in decol. solution (20% (vol/vol) of stock in PBS)	–	1.5 ml	1 d	8–9 d	2 h each wash	2 h	2 h + overnight	1–2 h	At least 12 h
Mouse pups (P0)	–	–	4.5 ml	1 d	4 d	2 h each wash	2 h	2 h + overnight	1 h	At least 3–4 h
Mouse whole head	If blood content is high: 1 d in decol. solution (20% (vol/vol) of stock in PBS)	2 d	50 ml	2 d	6 d	6 h each wash	12 h	12 h	1 h	At least 5–6 h
2-mm-thick pig pancreas slices	–	–	4.5 ml	1–2 d	7–10 d	2 h each wash	2 h	2 h + overnight	1 h	At least 3–4 h

[a]Timings are given for Chromotek nanobodies; increase the staining time as indicated in Table 1 for other nanobodies

Table 3 | Experimental pipeline of standard vDISCO versus mild-vDISCO for passive staining

Step	Standard passive-vDISCO solutions	Mild-vDISCO solutions	Temperature
Decolorization (optional)	Decolorization solution	Decolorization solution	RT or up to 37 °C
Washing	1× PBS	1× PBS	RT
Decalcification (optional)	Decalcification solution	Decalcification solution	RT
Washing	1× PBS	1× PBS	RT
Permeabilization	Permeabilization solution	Permeabilization solution	37°C
Staining	Staining solution with methyl-β-cyclodextrin and *trans*-1-acetyl-4-hydroxy-l-proline, + dye	Mild staining solution without methyl-β-cyclodextrin and without *trans*-1-acetyl-4-hydroxy-l-proline, + dye	37°C
Washing	Washing solution	Washing solution	RT
Washing	1× PBS	1× PBS	RT
Clearing	THF series, DCM, BABB (Table 2)	THF series, DCM, BABB (Table 2)	RT

Dyes with small size are ideal for vDISCO owing to their better tissue penetration ability. We found that the small nuclear dye propidium iodide (PI) efficiently labels all the nuclei of the cells of our samples with both passive-vDISCO and whole-body vDISCO[23]. Interestingly, PI tends to accumulate in tissues dense of cells from organs such as thymus, lungs and especially bone and its marrow, in this way being a good stain to highlight the internal organs and bones of the animal. Owing to this property and its red spectrum (excitation (Ex)/emission (Em) = 535/617) the PI can be used in multichannel imaging with nanobodies conjugated with far-red fluorophores: for example, in the neuronal reporter line Thy1-GFPM, PI was used as counterstain for organs and bones, while the nanobody was used to follow the trajectories of the nerves through the bones[23]. The TO-PRO-3 far-red (Ex/Em = 642/661) nucleus dye can be used instead of PI, but it shows a strong batch-to-batch variability in terms of staining performance: some batches provide very low signal. We do not recommend DAPI (Ex/Em = 350/470) as counterstain of big tissues, because its UV spectrum allows only superficial penetration of the light[52]. However, 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. Nanobodies show cross-compatibility with fluorescent proteins with a similar structure: for example, the anti-GFP can also bind (e)YFP, Venus, CFP and other GFP derivates, including eGFP, sfGFP etc., or the anti-RFP can also bind its derivates such as mRFP, mCherry, DsRed, etc. Further compatibility information can be found in the vendors datasheet: for example, the anti-RFP nanobody from the Chromotek company does not recognize tdTomato, while some clones from Nanotag do (R.C., unpublished observations), as also reported in Table 1.

We observed that fluorophores from the Atto and Alexa family give good performance with vDISCO (Table 1, Figs. 2 and 5–9 and Extended Data Figs. 2 and 3). If performing custom conjugation of the nanobody with a desired fluorophore (Fig. 7e and Extended Data Fig. 3c), different chemistries for the conjugation reaction might need to be tried (NHS, maleimide, enzymatic, etc.), depending on the sequence of the nanobody and the chosen dye: for example, it is known that Atto647N is a sticky dye that can cause unspecific staining of the tissue. Moreover, when lysine residues are present in the complementarity-determining regions of the nanobodies, site-directed chemistry should be favored to avoid the labeling affecting the binding capacity of the nanobody.

To exploit the deeper penetration of long-wavelength light, 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. Nanobodies show high performance variability as well: in our experience, diverse success in staining could be observed depending on the nanobody clone, the supplier, the conjugation strategy and even the batch of the same product (Fig. 7 and Extended Data Fig. 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 (Fig. 7a–e and Extended Data Fig. 3a–c) they showed variable results (e.g., Fig. 7a versus Fig. 7b), indicating a possible issue in the chemical bond between the nanobody and the fluorophore in our application. Therefore, some batches from

Chromotek require the use of mild–vDISCO (Tables 1 and 3, Fig. 7f–i, Extended Data Fig. 3d,e and 'Procedure' section). Nanobodies from Nanotag company were tested for vDISCO passive staining. 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, Fig. 7c and Extended Data Fig. 3a), and FluoTag-Q (Table 1, Figs. 7d and 9d–h and Extended Data Fig. 3b) made by a clone coupled exactly 1 to 1 with the fluorophore molecules. Fluotag-X4 provided high brightness but penetrated less (e.g., Figure 7c versus Fig. 7i and Extended Data Fig. 3a versus Extended Data Fig. 3e); on the other hand, FluoTag-Q gave excellent results for passive staining of microglia and pericytes expressing GFP (Figs. 7d and 9d–h), although it also showed less penetration power than the Chromotek nanobodies in the Thy1-GFPM line (Extended Data Fig. 3b versus Extended Data Fig. 3e). In general, Nanotag nanobodies demonstrated very high stability, without the need to use mild-vDISCO (Tables 1 and 3 and Fig. 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 complementarity-determining regions, the affinity and the epitope recognized. Nevertheless, the penetration performance can be increased by extending the incubation times for passive staining (e.g., 20–30% longer) and/or increasing the temperature up to 40 °C. We did not observe detrimental consequences of long incubations (up to 22 d). 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 in Thy1-GFPM brains (Extended Data Fig. 3c). Regarding the concentration, 0.08–0.14 μg ml^{-1} of nanobody is sufficient to stain a whole mouse, while 10× more concentrated nanobody was used for whole mouse brains processed with passive-vDISCO (Table 1). However, in both protocols the concentration can be adjusted on the basis of the expression of the fluorescent protein and the size of the sample.

Last, nanobodies must be validated before commencing the experiment to verify that the nanobody is able to sustain the staining and clearing conditions (Box 1). In general, nanobodies that do not normally give a good performance in standard immunohistochemistry and immunofluorescence 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

The clearing process starts after staining, and it is carried with a slightly modified version of 3DISCO[5,39], a straightforward and fast clearing protocol that can achieve high transparency. For both whole bodies and dissected samples, the clearing procedure consists of passive incubations of the samples with organic solvents. These solvents must not be perfused, since they can damage the pump components. In fact, organic solvents can often melt plastic; thus, plastic containers used for clearing must be checked for their resistance to these chemicals. In our experience, polypropylene from specific manufacturers stands organic solvents well (see 'Materials' section). Whole mice or big organs such as pig pancreas[37] 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.

Then, samples are incubated with an ascending series of dilutions of tetrahydrofuran (THF) to dehydrate, dichloromethane (DCM) to delipidate and a mixture of benzyl alcohol and benzyl benzoate (BABB, which replaces dibenzyl ether (DBE) of the original 3DISCO) to match the refractive index (RI) and reach transparency. The duration of each dehydrating incubation is highly flexible and depends on the size of the sample and its lipid content (for THF timings, see Table 2). The delipidation with DCM is optional but recommended with lipid-rich organs or whole bodies (for DCM timings, see Tables 2 and 4). All procedure timings (Tables 2 and 4) can be adjusted and extended on the basis of preliminary tests. With overweight animals, prolonging all the steps of clearing will improve the transparency.

Since synthetic fluorophores are highly stable in the clearing reagents[23], over-incubating the samples for multiple days in the clearing solutions (if, for example, left by accident) did not result in dramatic bleaching of the fluorescence signal. The level of transparency will probably increase over time with a longer incubation in the RI matching solution as more solution penetrates the sample. BABB can be replaced with DBE with comparable results, although we chose to use BABB because it

Table 4 | vDISCO active staining pipeline

		Adult mouse			Mouse pups		
Step	**Solution**	**Modality**	**Time**	**Temperature**	**Modality**	**Time**	**Temperature**
Boosting							
Washing	1× PBS	Active	2–3 h × 2 times	RT	Active	4–5 h	RT
Decolorization	Decolorization solution	Active	2 d (refresh when solution turns yellow)	RT	Active	3 d (refresh when solution turns yellow)	RT
Washing	1× PBS	Active	2–3 h × 3 times	RT	Active	4–5 h	RT
Decalcification	Decalcification solution	Active	2 d	RT	Active	2 d	RT
Washing	1× PBS	Active	2–3 h × 3 times	RT	Active	4–5 h	RT
Permeabilization	Permeabilization solution	Active	12 h	RT	Active	12 h	RT
Staining	Staining solution (permeabilization solution + dye)	Active	6 d	With infrared lamp (26–28 °C)	Active	4–6 d	With infrared lamp (26–28 °C)
Staining	Staining solution (permeabilization solution + dye)	Passive	2 d	37 °C or RT for nanobodies with stability issues (Table 1)	–	–	–
Washing	Washing solution	Active	2–3 h × 3 times	RT	Active	4–5 h × 2 times	RT
Washing	1× PBS	Active	2–3 h × 3 times	RT	Active	4–5 h	RT
Clearing							
Dehydration	50%, 70%, 80% THF in distilled water	Passive	12 h each dilution of THF	RT	Passive	12 h each dilution of THF	RT
Dehydration	100% THF	Passive	12 h × 2 times	RT	Passive	12 h × 2 times	RT
Delipidation	100% DCM	Passive	3 h	RT	Passive	3 h	RT
Refractive index matching	BABB	Passive	>12 h	RT	Passive	>12 h	RT

is slightly denser than DBE and therefore samples are less subjected to moving during imaging. BABB represents the storing solution as well, where samples can be left for months and years, and if at room temperature and protected from light, they still retain fluorescence signal[23] (Figs. 2 and 5). Nevertheless, we did not experience fading of the signal if stored at 4 °C.

Last, samples stained with vDISCO can be cleared with other clearing protocols such as iDISCO+, which is based on methanol dehydration. This finding is interesting if the advantages of other clearing methods are desired: for example, iDISCO+ clearing is known to shrink the sample less compared with 3DISCO[53].

Imaging

The samples will stay transparent as long as they are completely submerged in the RI matching solution; thus, this solution must constitute the sample mounting medium for imaging as well.

Ideally the microscope's objectives should provide decent resolution with the highest possible numerical aperture (NA) and with the longest possible working distance (WD), to cover the whole thickness of the sample. The objectives can be air lenses or immersion lenses; the latter should be optimized for the RI of the RI matching solution or of the immersion medium, to reduce optical aberrations and increase the resolution.

An epifluorescence microscope such as the Zeiss AxioZoom EMS3/SyCoP3 (Extended Data Fig. 5a–d), which can support a long WD 1× air objective[1,23] ideal to quickly assess the success of the staining (e.g., while testing slices) or to obtain whole-body 2D images in 10–15 min.

On the other hand, light-sheet microscopes[15] (Extended Data Figs. 6, 7 and 8) are ideal to achieve 3D high-resolution imaging of cleared samples, because in these systems the light-sheet illuminated focal plane is simultaneously captured with a scientific complementary metal oxide semiconductor (sCMOS) camera, allowing very fast imaging and low photobleaching.

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

The Ultramicroscope II has an imaging chamber of 8 × 8 × 3.5 cm with a standard sample holder travel range of 1 ×1 × 1 cm (in the *XYZ* axes), which we increased to 1.1 cm in *z* by a custom-made stage (available from the manufacturer). This microscope was used to create the whole-body neuronal projection map of a Thy1-GFPM mouse[23], by using a 1× air objective coupled with a zoom body kept at 0.63×. With these settings the field of view (FOV) was 2 × 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 together. For labs owning the Ultramicroscope II, we suggest this strategy to image whole mice or samples bigger than the travel range, although the process of mounting the sample can be quite complicated and the entire imaging step can take 1–2 weeks (Extended Data Fig. 6a–j).

Therefore, we suggest using light-sheet microscopes with bigger sample holders and imaging chambers such as the LaVision-Miltenyi Biotec Blaze light-sheet microscope, which is also optimized for large samples, including whole mouse bodies[1] and intact human kidneys[37]. Our Blaze system possesses an elongated customized sample holder that can travel 4 × 9 × 5 cm in the *XYZ* axes and a large imaging chamber of 25 × 7 × 9 cm (both available from the manufacturer). Moreover, it works with a dipping 1.1× objective that has a FOV of 1.2 × 1.2 cm. With this microscope, an entire mouse can be imaged with one tiling scan in just 2–3 d, although we recommend imaging the body in two tiling scans with an overlap: the first scan covering one side and the other scan after flipping the animal. The two scans will be fused together in the end. This strategy will allow achieving the best resolution for both sides, overcome the limited WD of the objective and the possible incomplete transparency of the samples.

The Zeiss Lightsheet Z.1 is a light-sheet microscope with a closed chamber optimized for single organ or small organism imaging (Extended Data Fig. 8). It has an imaging chamber of 1 × 1 × 2 cm, a sample holder travel range of 1 × 5 × 1 cm and a rotation stage for multi-angle scans to achieve isotropic resolution in 3D (Fig. 5).

We used the Lightsheet Z.1 and the Ultramicroscope II to capture scans of whole mouse organs (e.g., lungs, heart and brain) with higher magnification and NA objectives such as a 5× objective for the Z.1 (Fig. 5), a 4× objective (Figs. 6b–f, 7 and 9d–h and Extended Data Fig. 3) or a 12× objective[23] (Fig. 9a–c) for the Ultramicroscope II. We 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).

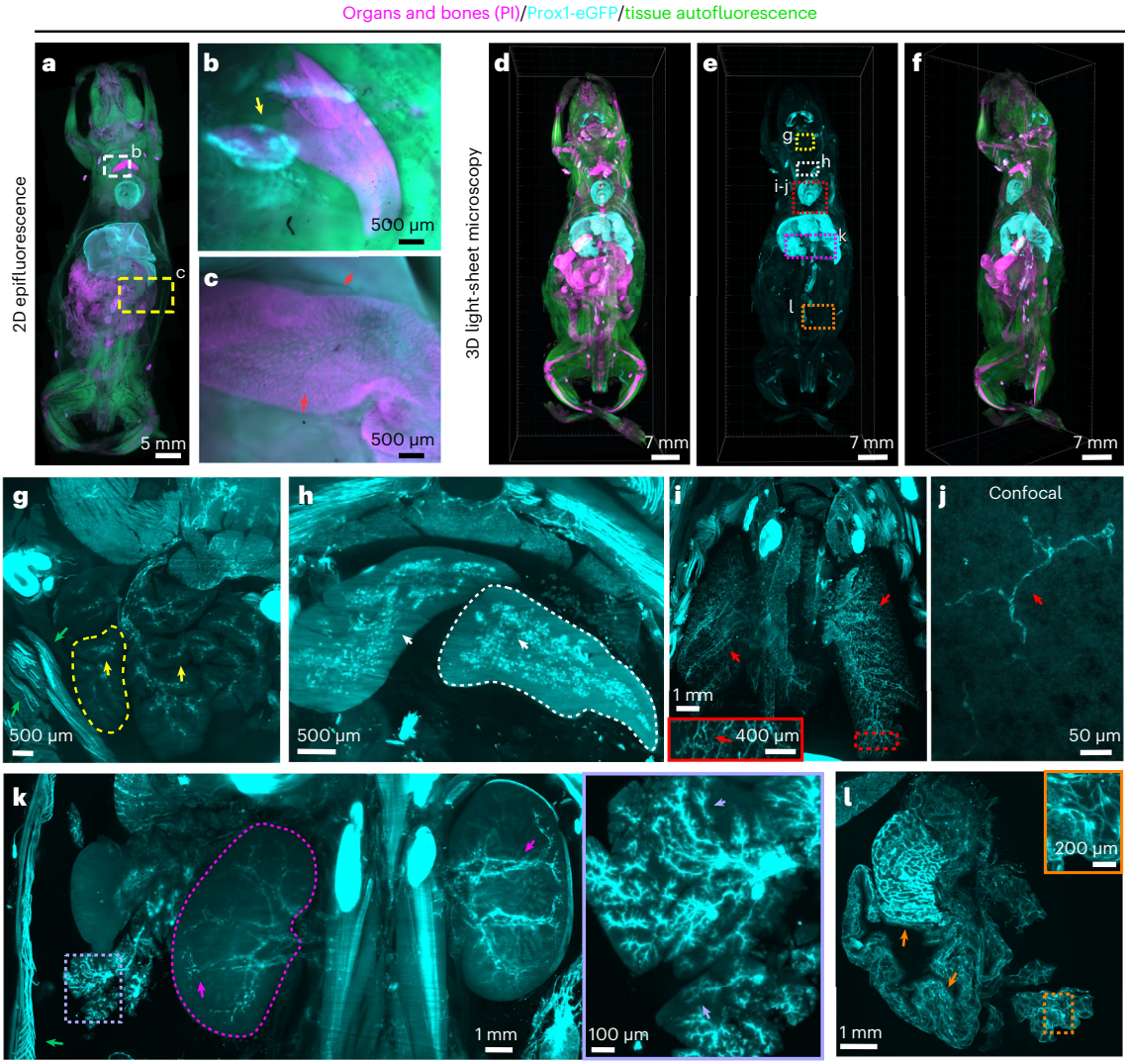

Fig. 8 | Prox1-EGFP whole-body scan taken with epifluorescence, light-sheet and confocal microscopy. a–c, 2D epifluorescence microscope imaging of a Prox1-EGFP mouse after vDISCO pipeline (**a**). Zoomed-in views (**b** and **c**) of the dashed regions in **a** showing the thymus (yellow arrow) and the intestine, respectively. Red arrows in **c** indicate intestine villi. **d–f**, 3D reconstruction of the same mouse imaged with light-sheet microscopy in dorsal (**d** and **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**. In **g** and **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. In **g** and **i–l**, Prox1-EGFP+ signal is visible as elongated structures in the body regions including some muscles (**g** and **k**, green arrows), in the body region showing the lungs (**i** and **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** are shown in ventral view, while **k** 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** versus **h**) and in the intestine (**c** versus **l**). Prox1-EGFP+ signal is shown in cyan in all the panels. In **a–c**, **d** and **f**, organs and bones are labeled by PI and shown in magenta while tissue autofluorescence is shown in green. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.

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] microscopes (Extended Data Fig. 5e–g), being aware that the commercial systems of these microscopes are normally coupled with high-NA objectives with therefore limited WD. Here, samples processed with vDISCO were imaged with the laser scanning confocal microscope Zeiss LSM 880 by using a Leica long WD 25× objective mounted on a custom mounting thread (external M27x0.75 to internal M25x0.75 adapter ring, which has no optics and does not modify any optical parameter; available from the manufacturer)[23] (Figs. 6a, 8j and 9h). However, the use of objectives from a different manufacturer can

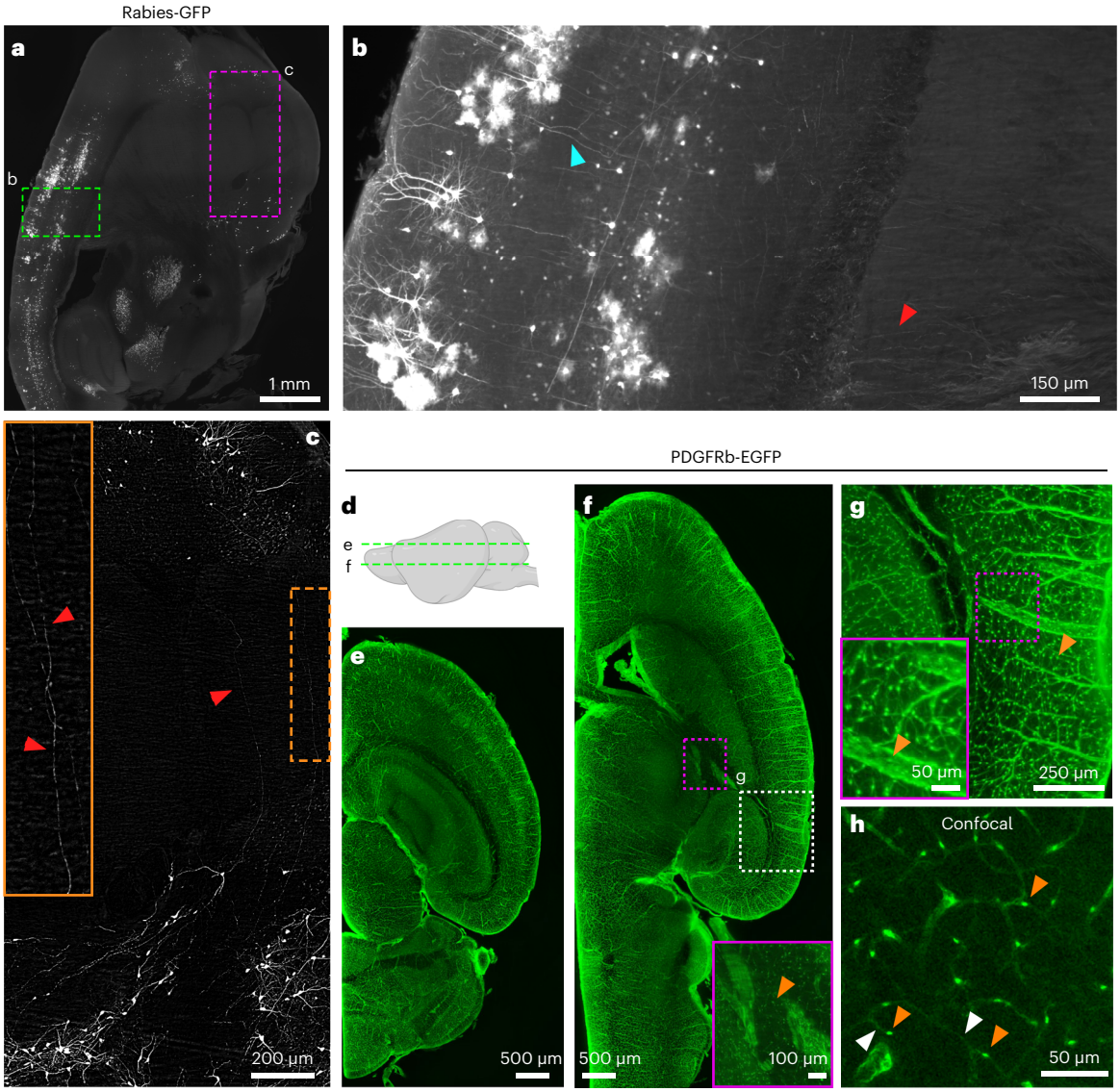

Fig. 9 | Virus tracing and pericytes in the brain processed by vDISCO. a–c, Light-sheet microscope 2D images of a brain from an adult Emx1-Cre × RΦGT mouse injected with EnvA-pseudotyped G-deleted rabies virus expressing GFP (SADB1969) into the neocortex and processed with vDISCO. The images show the virus traced neurons with single axons visible in the cortex (**b**, cyan arrowhead) and in the striatum (**b**, red arrowhead) in the raw data and in the postprocessed image (**c**, red arrow-heads). The postprocessing was performed with sharpening of the signal, noise 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**): two images (**e** and **f**) corresponding to optical slices indicated in **d** are shown. Single EGFP^{+} pericytes are visible throughout the brain and wrapping blood vessels (**f** and **g**, orange arrowheads). In **h**, in confocal imaging the pericyte somas (orange arrowheads) and their fine processes (white arrows) are visible. For both virus traced and pericyte samples, similar results were obtained in at least three independent brains. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was moderate for virus tracing and low for pericyte labeling.

have several drawbacks such as chromatic, spherical aberration and axial distortions, due to the mismatch in focal lengths and RI. We recommend the users choose the objective on the basis of their applications and, if possible, ask the microscope manufacturer about eventual drawbacks and customizations.

Last, the mesoSPIM[54] 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 min per mouse brain at 5 µm sampling versus 3 h of the Ultramicroscope II, thus 12× faster), owing to the FOV of 2–20 mm, the travel range of 52 × 52 × 102 mm and the 360° rotating sample holder for multiview imaging[54]. With its large travel range, the mesoSPIM is capable

of imaging the whole mouse central nervous system[54] without remounting the sample. Since the mesoSPIM is fully customizable, a version tailored for imaging whole mice with no need of remounting (Extended Data Fig. 2) was built by updating the published version. The updated microscope has a larger (100 × 200 × 100 mm) *XYZ* travel range, uses imaging cuvettes of 30 × 30 × 120 mm or 40 × 40 × 120 mm and has an easy strategy to mount the samples on the basis of self-centering magnets (Extended Data Fig. 7). To reduce the number of required tiles while retaining micrometer-level sampling, an integrated CMOS camera with 3.75× more pixels compared with the previous version (15 MP versus 4 MP) was added in the system. The modified mesoSPIM allows us to perform two channel acquisitions of whole mice with 4.7 × 4.7 × 10 µm sampling within 9 h.

Regarding the data collection, raw images are collected as grayscale TIFF stacks with a pixel intensity range of 16 bits in order to resolve details in images with high contrast in intensity. Scans are saved as Zeiss CZI files if from Zeiss microscopes. In the latter case, the single images constituting a stack can then be exported as TIFF files as well.

Data processing and analysis

The data generated by vDISCO span from hundreds of gigabytes for individual organs to several terabytes for a single mouse. To handle these data, first, the size can be reduced by simply compressing the raw TIFF files with a lossless compression algorithm: we suggest Lempel-Ziv-Welch (LZW)[55], which is widely supported by common scientific software. Some commercial software for image rendering and analysis might have their own data structure that includes streams of compressed data (e.g., HDF5 in the IMS format from Bitplane Imaris or the SIS format of Arivis Vision4D). We convert our stacks to these structures for a fast loading of the files while also keeping a copy of the raw data.

Since almost all our data were generated as tiling scans, 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 on the basis of visual inspection and merged together with Adobe Photoshop CS6 using its 'Photomerge' function[56–58]. It should be noted that this procedure converts the 16-bit images to 8-bit RGB images irreversibly in addition to disabling single-channel viewing; therefore, it should be used only for the purpose of visualization of representative data. Alternative tools that work on 16-bit images include Fiji stitching plugins such as the more advanced 'BigStitcher'[59], which provides the advantages of flexible tile positioning, handling missing tiles, being open source and scriptable. Tiling scans saved in CZI format were automatically stitched by the Zen (Zeiss) acquisition software immediately after completing the acquisition. Tiling scans saved as TIFF stacks were stitched using Fiji[60] (ImageJ)'s stitching plugin[61] (Supplementary Fig. 1); optionally, the removal of acquisition errors/shifting can be done by using Fiji's TrakEM2 plugin and Imglib2library. Alternatively, tiling errors in the *XY* and *Z* dimensions can be corrected by using TeraStitcher[62] (v.1.10; https://abria.github.io/TeraStitcher/). Whole-mouse stitching/reconstruction processes can be heavily impaired by insufficient RAM. However, this can be overcome by using read on demand and caching implementations/code as we did in the Volume Fusion module of the Vision4D (Arivis) to stitch the whole mouse scan (Supplementary Figs. 2 and 3).

Data visualization and simple processing including filtering, equalization, histogram adjustments and contrast enhancement can be performed with several software tools: Fiji, Amira (FEI Visualization Sciences Group), Imaris (Bitplane) and Vision4D. The latter three were also used for 3D rendering of the scans. In particular, we used Fiji 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, software such as Neurolucida[63] or NeuroGPS-Tree[32] are available to analyze the morphology and quantify the features of the neurites[23] in neurons, or ClearMap[53] to automatically count, register and annotate cells with distinguishable round soma to the Allen Mouse Brain Atlas. All mentioned software tools offer 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. 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. Second, although some of these tools can perform automated analysis, all of them rely on traditional analytical approaches based on explicit preprocessing and recognition with filters, of which parameters must be adjusted by the human operator based on the characteristics of each individual dataset[53]. Since fully automated data analysis is greatly

desired owing to the complexity and the amount of data produced by the vDISCO pipeline, new computational tools based on artificial intelligence have now started solving these bottlenecks. In fact, the high-contrast images that vDISCO is able to provide represent suitable data that can be analyzed using deep learning algorithms. These algorithms are able to learn from the information provided by human experts and adjust their criteria in a dynamic and autonomous way depending on the characteristics of the new dataset[64,65]. This results in a reliable processing of large information in a very fast, accurate and unsupervised manner[66]. For instance, we developed a deep learning algorithm called DeepMACT to be run on vDISCO cleared animals to automatically detect, segment, quantify and annotate single metastasis in intact mice of cancer models[1].

Computational power, data storing and backing up

For the analysis of data generated via vDISCO, we used different standalone workstations: an HP Z820 with 196 GB RAM and with eight-core Xeon processor and nVidia Quadro k5000 graphics card, an HP Z840 dual Xeon 256 GB DDR4 RAM and with nVidia Quadro M5000 8GB graphic card, and an 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. In general, RAM should be at least 256 GB and at least one SSD drive should be dedicated to the OS. In addition, we used multiple Promise Technology Pegasus2NAS (network attached storage with 96 TB capacity) devices to store the data collected daily 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 adopted in private sectors and in some labs[67]. 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, 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 redundant array of independent disks (RAID) configuration for speed and redundancy (depending on implemented local policy).

Materials

Reagents

Samples

Samples should be chosen from animals expressing fluorescent proteins that have been shown to be compatible with a particular nanobody according to the nanobody data sheet. The sample can express the fluorescent protein also with viral strategies. Alternatively, a sample coming from animals transplanted or injected with fluorescent-protein-expressing cells can be used. The following mouse lines are examples of suitable strains to use: Thy1-GFPM[36] (and Thy1-YFPH), CX3CR1$^{GFP/+}$ (B6.129P-Cx3cr1tm1Litt/J; Jackson Laboratory strain code 005582[27]), 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 d, and an adult Emx1-Cre × RΦGT mouse[68,69] injected with EnvA-pseudotyped G-deleted rabies virus expressing GFP (SADB19 (ref. [70])) 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 conforming to the institutional guidelines indicated in the figure legends.

Reagents for sample preparation

- 10× phosphate-buffered saline (PBS), 0.1 M, 10× stock solution (Invitrogen, cat. no. AM9625)
- Double-distilled water (ddH_2O)
- MMF triple combination anesthetics: midazolam, medetomidine and fentanyl (1 ml per 100 g body mass for mice; intraperitoneal) (Fentanyl, Panpharma, 0,5mg/10ml, Midazolam, Braun, 5mg/5ml, Dorbene vet, Zoetis, 1mg/ml) ▲ **CRITICAL** Follow the regulations of your institution regarding the drugs used for anesthesia.
- Heparin, 5,000 U/ml (Ratiopharm, cat. no N68542.03)
- 4% paraformaldehyde (PFA) in 1× 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.

Reagents for decolorization, decalcification and immunostaining

- Urea (Carl Roth, cat. no. 3941.3)
- 10× PBS
- ddH_2O
- Quadrol, also known as *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)
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. 71687)
- Goat serum (Gibco, cat. no. 16210072)
- Methyl-β-cyclodextrin (Sigma, cat. no. 332615)
- *trans*-1-acetyl-4-hydroxy-L-proline, also known as *N*-acetyl-L-hydroxyproline (Sigma-Aldrich, cat. no. 441562)
- PI (Sigma-Aldrich, cat. no. P4864 or Invitrogen cat. no. P3566)
- Nanobody (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 system One system (Leica, model Perfusion One), used for the initial fixation step ▲ **CRITICAL** This perfusion system can be replaced with other perfusion methods/set ups.
- Disposable 30 ml syringes (any; we used B. Braun, cat. no. 4616308F)
- Disposable 1 ml syringes (B. Braun, cat. no. 9166017V)
- Disposable 1 ml syringes with 25 G needle (B. Braun, model Injekt-F, cat. no. 9166033V)
- Micro-Fine Ultra needles 12.7 mm, 0.3 mm 29 G (BD, cat. no. 09372884)
- Microlance 3 needles 0.3 mm, 13 mm 30 G (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)
- (Optional) Hair removal cream (Veet, Hair Removal Cream Normal)
- 5 ml tubes (Eppendorf, cat. no. 0030 119.401) ▲ **CRITICAL** These particular tubes are resistant to the clearing solutions.
- 50 ml tubes (Thermo Fisher Scientific, cat. no. 339653) ▲ **CRITICAL** These particular tubes are resistant to the clearing solutions. 50 ml tubes from Falcon brand (cat. no. 352070) are also resistant to the clearing solutions, but prone to leak BABB. 15 ml Falcon tubes are not recommended owing to the easy-to-break lid.
- 24-Multiwell plates (Falcon, cat. no. 353504) ▲ **CRITICAL** Multiwell and cell culture plastic are generally made from polystyrene, which is not resistant to the clearing solutions. Use these containers 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), resistant to the clearing solutions
- Pipette boy (any; we used 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 (we used Corning Costar 25mL Stripette, cat. no. 4489) ▲ **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; we used VWR, cat. no. 391-2025)
- 1 L glass beakers (any; we used DURAN, cat. no. 211075409)
- 1 L glass bottles (any; we used DURAN, cat. no. DU218015455)
- 2 L glass bottles (any; we used DURAN, cat. no. 218016357)
- 500 ml glass bottles (any; we used DURAN, cat. no. DU218014459)
- (Optional) One Büchner flask or vacuum glass flask with a rubber stopper (any; we used VWR, 511-0047)
- Polytetrafluoroethylene (PFTE) also known as Teflon covered magnetic stirring bars (any; we used IKAFLON, cat. no. IKAA4488700)
- Hot magnetic stirrer (any; we used IKA, model RCT basic B-5000, cat no. 0003810000)
- Aluminum foil (any; we used Korff AG, cat.no. 60050)
- Parafilm (Bemis, cat. no. PM-992)
- Transparent sticky tape (any)
- Electrical black insulation sticky tape (any, we used SoundOriginal, available from Amazon.com)
- 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 labeling pens (any; we used Edding, cat. no. 140003)
- Any sample and tube holders with the shape as in Fig. 3a
- 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 3–5 mm diameter (Omnilab, cat. no. 5434482 or 8700-0406)
- PVC tubing (Omnilab, cat. no. 5437920)
- Infrared lamp (Beuer, cat. no. IL21)
- pH meter (any; we used WTW, model pH7110)
- Vibratome (any; we used Leica, model VT1200S)
- Incubator (Memmert, model UN160), or any incubator that can hold a shaker inside at 37 °C
- 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

Zeiss AxioZoom EMS3/SyCoP3 fluorescence stereomicroscope with

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

LaVision-Miltenyi BioTec 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 0.63–6.3×)
- Olympus revolving zoom body unit (U-TVCAC)
- 1× air objective (Olympus MV PLAPO ×1/0.25 NA, WD 65 mm)
- 2× immersion objective (Olympus MVPLAPO2XC/0.5 NA, WD 6 mm)

- 4× immersion objective (Olympus XLFLUOR340 ×4 corrected/0.28 NA, WD 10 mm),
- 20× immersion objective (Zeiss ×20 Clr Plan-Neofluar/1.0 NA, WD 5.6 mm)
- 12× objective (LaVision-Miltenyi BioTec MI PLAN ×12/0.53 NA, WD 10 mm with dipping cap for organic solvents)

LaVision-Miltenyi Biotec 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 (Extended Data Fig. 6k-r)
- Customized large imaging chamber (25 ×7 × 9 cm length–width–height)
- 1.1× objective (LaVision-Miltenyi BioTec MI PLAN ×1.1/0.1 NA, WD 17 mm)
- 12× objective (LaVision-Miltenyi BioTec MI PLAN ×12/0.53 NA, WD 10 mm with dipping cap for organic solvents)

Zeiss Lightsheet Z.1 light-sheet microscope

- Detection objective: 5× air objective (Zeiss EC Plan-Neofluar ×5/0,16 NA, WD 10.5 mm) compatible with water-based and clearing solutions $n = 1.45$
- Illumination: Zeiss LSFM ×5/0.1 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: 5× clearing chamber for sample size of 1 × 1 × 2 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* and 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 0.9× magnification and lens clamp
- Teledyne Photometrics Iris 15 camera with 15-megapixel resolution
- Optomechanical architecture and electronic controlling system of mesoSPIM (https://github.com/mesoSPIM/mesoSPIM-hardware-documentation)
- Custom 40 mm × 40 mm × 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

- 25× water-immersion objective (Leica Fluotar Visir, ×25/0.95 NA, WD 2.5 mm) mounted with a custom mounting thread

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[60] (ImageJ2, v.1.51, https://fiji.sc/)
- (Optional) TeraStitcher[62] (v.1.10, https://abria.github.io/TeraStitcher/)
- (Optional) Total Commander (v. 8.52a ×64, https://www.ghisler.com/)
- Photoshop CS6 (v. 13.0, Adobe)

Box 2 | Clearing reagents toxicity and safety issues

Of the solvents used for vDISCO, only a few represent a health issue for the operator, and only if they are not handled properly. In particular, according to PubChem, THF represents a hazard owing to its explosivity and high flammability, and irritation caused to the eyes and skin. It is classified as a moderate toxic agent in acute exposure and suspect carcinogen in chronic exposure at least in rodents, while data in humans are limited. In the same database, DCM is reported as a health hazard in certain conditions: it is inflammable, causes severe irritation to the eyes and to the respiratory tract, and is responsible for moderate skin irritation up to chemical burns under prolonged exposure. DCM has mutagenic and carcinogenic effects in animals, while no confirmation of such effects was obtained in humans. The inhalation of THF or DCM can cause headaches, dizziness, nausea and anesthesia. Handling and storing DCM and THF must follow dispositions for hazardous, inflammable, explosive and toxic substances; in particular, handling must be performed in fume hoods, while wearing safety goggles, nitrile gloves (preferably double layer) and a lab coat.

According to the safety data sheet, benzyl alcohol has fulfilled the 'Safer Choice Criteria' as a relatively safe compound. It can irritate skin, eyes and respiratory tract. It is not listed among the carcinogenic agents.

Finally, benzyl benzoate is considered an irritant and a harmful substance if swallowed. According to the Hazardous Substances Data Bank, it is an irritant to the eyes and skin. There is no evidence of its carcinogenic effects in either animals or humans. It is considered an environmental hazard that can affect aquatic life. Both benzyl alcohol and benzyl benzoate are combustible but not inflammable and explosive. The prolonged inhalation of benzyl alcohol or benzyl benzoate can cause coughing and dizziness. According to the US Food and Drug Administration, these compounds can be used in minimum quantity as food additives or as cosmetic ingredients for human use and consumption. Handling both benzyl alcohol and benzyl benzoate should be performed in a well-ventilated environment, possibly in a fume hood or under an aspiration system, and wearing safety goggles, double nitrile gloves and a lab coat. Operators must follow all the safety and disposal dispositions for all the chemicals used in vDISCO. More information and data are available at https://pubchem.ncbi.nlm.nih.gov/.

- ClearMap[53],
- DeepMACT[1]
- NeuroGPS-Tree[32]

Reagent setup

! 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 2.

1× (0.01 M) PBS

Dilute 10× PBS with ddH_2O to obtain 1× PBS. 1× PBS solution can be stored at room temperature (18–25 °C) for several months.

Heparinized PBS

Dilute the stock solution of heparin (5,000 U/ml) into 1 L of 1× PBS to reach final concentration of heparin 25 U/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 several weeks at 4 °C.

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 (see 'Materials' section). Otherwise, any 4% PFA in 1× PBS for perfusion is usable. **! 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 1× 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. It can be stored at RT for several months. **! CAUTION** This solution is toxic. Avoid contact with skin and eyes.

Decolorization solution

Stock solution preparation: in a 1.5–2 L beaker mix 25 wt% urea, 25 wt% Quadrol and 15 wt% Triton X-100 in ddH_2O. To facilitate the dissolution, stir 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 RT. The stock solution can be stored for 2–3 months at RT. 1–2 L of the stock is sufficient for one or two animals. **▲ CRITICAL** Avoid boiling the stock solution when stirring.

Owing to the high amount of detergent, be careful not to generate too many bubbles or foam when mixing.

Final decolorization solution preparation: dilute the stock solution 20–30% (vol/vol) with 1× PBS (for whole-body active vDISCO, use 25%). Mix well until the final solution turns homogeneous 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.

Decalcification solution

Dissolve 10% (wt/vol) EDTA in 1× PBS by stirring with a magnetic stirring bar and by adding NaOH in powder until no aggregates are visible and the pH reaches ~8. The NaOH is necessary to help the EDTA dissolve. Heating the solution is not recommended, because the reaction already generates heat and the pH should be measured when the solution has cooled down to RT. 1 L of solution is enough for whole-body vDISCO of three or four 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.

Permeabilization/staining solution

The permeabilization/staining solution is used in both the 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 1× PBS. 600–700 ml is enough to process one animal with whole-body vDISCO. ▲ **CRITICAL** Prepare fresh solution before starting the experiment, it can be stored for a few days at 4 °C.

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 1× PBS. 600–700 ml is enough to process one animal with whole-body vDISCO. ▲ **CRITICAL** Prepare fresh solution before starting the experiment; it can be stored for a few days at 4 °C.

Dehydration solutions

Transfer 500 ml (this amount might be decreased to 100 ml for small samples) of 100% THF from the stock bottle into a glass bottle to limit repetitive opening of stock bottles. This solution will be used as the 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 ddH2O. 250 ml of each dilution is sufficient to treat one whole body. All solutions can be stored in glass bottles with a solvent-resistant lid at RT in the dark for several weeks. ! **CAUTION** For information on THF toxicity, see Box 2. THF tends to form peroxides over time, being an explosive hazard if stored for months or years. Purchase THF in max 2 L bottles and avoid stocking up unused THF in the lab for long periods. THF containing a stabilizer such as 250 ppm BHT (see 'Reagents' section) has reduced formation of peroxides. Both open and unopened/new bottles of THF should be kept at RT 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 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 tissue 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. For further information about toxicity and handling of DCM, see Box 2. Store all DCM solutions in the dark and in well-sealed bottles at RT in a safety cabinet for solvents.

Refractive Index matching solution: BABB

Mix one volume of 100% benzyl alcohol with two volumes of 100% benzyl benzoate. 300–350 ml of BABB is sufficient for one animal. BABB can be stored for some months at RT in the dark in glass bottles sealed with lids resistant to solvents. ! **CAUTION** For information on the toxicity of BABB components, see Box 2.

Equipment setup

Transcardial-circulatory perfusion system

In whole-body active vDISCO, the setting up of the transcardial-circulatory perfusion system is required for decolorization, decalcification and staining, and it is established by using the Ismatec peristaltic pump as shown in Fig. 3. This pump can hold four pumping channels with one setup/tube per channel (Fig. 3b, left); therefore, four 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 (Fig. 3b, right and Fig. 3c, blue arrow). Insert the hose tubing connectors at both ends of the reference tube (Fig. 3c, red dashed rectangles). Through the tubing connectors, connect the reference tube with two additional PVC tubes at each side of the reference tube (Fig. 3c, magenta arrows), to extend the total length of the reference tube. Use parafilm to seal the connecting parts (Fig. 3c, 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 (Fig. 3c, yellow insert and Fig. 3d). 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** All the connections must be 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 injecting the MMF triple combination 1 ml per 100 g 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.

(a) If using the Leica Perfusion One system to perfuse the animal:

(i) Fill one of its pumping tanks with RT heparinized PBS (alternatively ice cold) and a second pumping tank with RT 4% (wt/vol) PFA (alternatively ice cold).

(ii) Start pumping for 1–2 min with the heparinized PBS to fill up the pumping tube and to push out all the air bubbles.

▲ **CRITICAL STEP** Avoid formation of air bubbles in the tubing because they can impair the subsequent circulation of the solutions (for fixation and later for staining) in the vasculature of the animal.

(b) Other possible perfusion methods:

(i) Use an electric peristaltic pump.

(ii) Manually push 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.

2 At RT, intracardially perfuse the anesthetized animal at pressure ~110 mmHg with the heparinized PBS for 5–10 min until the blood is flushed out.

3 Switch the perfusion with the 4% PFA solution for 10–20 min 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 (Extended Data Fig. 1a, cyan dashed line) and the perfusate drains clear from the right atrium. Moreover, the animal should become rigid and stiff starting from the second or third minute of perfusion with 4% PFA (usually after 25 ml of PFA).

? TROUBLESHOOTING

Passive-vDISCO staining ● Timing 2–23 d (excluding the optional decolorization and decalcification treatments: for timing details, see Table 2).

4 After perfusing with 4% PFA, dissect out the tissue or organ of interest from the animal.

▲ **CRITICAL STEP** Since the gut content cannot be cleared, the gut should be emptied at this stage either by flushing the content through small incisions made in the tissue with a syringe filled with 1× PBS or by gently and manually squeezing it out through the incisions (Extended Data Fig. 1b and Supplementary Videos 1 and 2).

5 Post-fix the dissected organs/tissues in appropriately sized tube in 4% PFA overnight at 4 °C.

▲ **CRITICAL STEP** The sample must be completely immersed in at least five sample volumes of 4% PFA solution. Avoid overfixation because it can increase the tissue autofluorescence. Label the tubes

with solvent-resistant ink (permanent pen, graphite pencil) and further cover the label with transparent tape, because all the solutions that will be used from now on can dissolve the labels.

6 Set up a rocker or a shaker that can hold the sample containers for gentle shaking. Set an incubator that must be able to hold the rocker or the shaker for the incubations at 37 °C.

7 After post-fixation, wash the samples with 1× PBS three times for 20 min for smaller samples, up to 2 h for the larger samples (Table 2), at RT and with gentle shaking.

PAUSE POINT Washed samples can be stored at 4 °C in PBS for up to 4 weeks or in PBS + 0.05% sodium azide for up to 1–2 years.

CAUTION Do not use samples where bacteria or fungal contamination is observed in the storing solution.

8 (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).

9 (Optional) Straighten dissected spinal cords or whole central nervous systems (brain and spinal cord) with the strategy described in Extended Data Fig. 4 by using plastic Pasteur pipettes cut in half and fine needles.

10 (Optional) Decolorization. For samples that cannot be efficiently flushed from blood in the PBS/PFA perfusion step (e.g., spleen or liver), perform the decolorization step by incubating the sample in the decolorization solution at RT with gentle shaking until the heme is eluted out. The sample will turn a lighter color, and the solution will turn yellow (Table 2). For a faster decolorization, the incubation can be performed at 37 °C. Refresh the decolorization solution two to three times (e.g., every 3–8 h) until the sample appears lighter in color and the solution does not turn yellow anymore.

CAUTION This treatment can reduce the overall tissue clearing performance of the sample; hence, carefully adjust the timing of this step and the concentration of the decolorization solution (Table 2). Preliminary tests with different timings and concentrations can help optimize the decolorization step.

11 Wash thorough at least five times with 1× PBS at RT to eliminate any traces of decolorization solution.

PAUSE POINT One of the washings in PBS can be extended to overnight.

12 (Optional) Decalcification. Decalcify samples (e.g., bones) by incubation in the decalcification solution at RT with gentle shaking until the sample becomes soft and flexible.

CRITICAL STEP The decalcification step can decrease the staining performance.

13 Wash five times with 1× PBS at RT to eliminate any traces of decalcification solution.

PAUSE POINT One of the washings in PBS can be extended to overnight.

14 Permeabilization. Treat the sample with the permeabilization solution (adjusted to sample size) at 37 °C with gentle shaking for 3 h to 2 d (for timing details, see Table 2).

15 Staining. Incubate the sample in the staining solution containing the dyes of interest at 37 °C with gentle shaking and protected from light for 3 h to 21 d. 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 Tables 1 and 2.

CRITICAL STEP For nanobodies that have stability issues as indicated in Table 1, mild-vDISCO protocol should be used: at this step, wash the permeabilized samples with the washing solution 30 min three times at RT. Then incubate in the mild-staining solution to which the dyes were added (Table 3). The sample containers must be tightly sealed (e.g., wrapping the lid container with parafilm) to prevent evaporation.

CRITICAL STEP The nanobody must be previously validated (Box 1).

16 Wash the sample with the washing solution 3 times (adjusted to the sample size) at RT, protected from light and with gentle shaking for 10 min to 1 h.

PAUSE POINT One of the washing steps can be extended to overnight.

17 Wash the samples with 1× PBS three times for 10 min to 1 h (adjusted to sample size) at RT, protected from light and with gentle shaking.

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

Whole-body active vDISCO staining ● Timing 12–16 d

Sample preparation

18 After the PBS–PFA perfusion of the animal, remove the skin and the eyes (Extended Data Fig. 1c; see also Table 4).

CRITICAL STEP Since the gut content cannot be cleared, the gut should be emptied at this stage either by flushing the content through small incisions made in the tissue with a syringe filled with 1

PBS or by gently and manually squeezing it out through the incisions (Extended Data Fig. 1b and Supplementary Videos 1 and 2). For clearing the non-nude whole mice with intact skin, remove the hair by shaving it off with a razor blade or by applying a commercial hair removal cream (Extended Data Fig. 1d).

19 Wash the whole body extensively with PBS to clean the body from hair and digested food as much as possible.

▲ **CRITICAL STEP** A large amount of hair and gut content residues can clog the pumping system that will be used later in the staining procedure.

20 Post-fix the specimen in 4% PFA at 4 °C in 50 ml tubes or bigger plastic containers for 1 d.

▲ **CRITICAL STEP** The sample must be completely immersed in the 4% PFA solution. Avoid overfixation because it can increase the tissue autofluorescence.

21 After post-fixation, wash the samples with 1× PBS three times each at RT and with gentle shaking for 1 h.

■ **PAUSE POINT** Washed bodies can be stored at 4 °C in PBS for up to 4 weeks and in PBS + 0.05% sodium azide for up to 1–2 years.

! **CAUTION** Do not use samples where bacteria or fungal contamination is observed.

Sample and perfusion system setup

22 Put the body of the animal in the 300 ml glass chamber (Fig. 3e) and place it close to the peristaltic pump (Fig. 3f).

23 Place the sucking end of the pumping tube inside the glass chamber until the tip touches the bottom of the chamber (Fig. 3e, bottom, black arrow).

24 Fill the chamber with 1× PBS with an amount that can cover the body.

25 Start the pumping for 2–3 min to make sure that the entire tube is filled with PBS with no air bubbles.

26 Using the sample/tube holders from Fig. 3a, fix the whole pumping tube with an angle that has the perfusion needle directed into the glass chamber (Fig. 3f).

27 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 (Fig. 3g,h).

▲ **CRITICAL STEP** Be extremely gentle pushing the needle inside the hole. Do not force the needle and do not push it too hard or too deep to prevent damaging or breaking the tissue around the hole or the heart. The hole must be just big enough for the blunt tip of the perfusion needle to get in. If the hole becomes too big, the perfusion solution can immediately leak out from the hole, resulting in insufficient circulation in the vasculature.

? TROUBLESHOOTING

28 (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 one to two 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 (Fig. 3i, left). Let the glue dry for a few minutes (Fig. 3i, right). Then, cover the animal back with 1× PBS.

▲ **CRITICAL STEP** 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 the pressure does not seem to be kept well while pumping (e.g., a lot of flow is coming out directly from the hole), then the glue can be added just before the staining step (Step 39).

? TROUBLESHOOTING

Decolorization

29 Set the peristaltic pump with a pumping rate of 45–60 rpm, which is 160–230 mmHg.

30 Start the pumping and perfuse the animal with the 1× PBS contained in the chamber for 2–3 h two times (one washing with PBS can be left overnight) at RT. 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 from bursts when handling the pumping system. You may adjust the pressure on the basis of the feedback from the body of the animal (smaller animals require less pressure). When the pressure is too high, it can damage the vasculature by creating leaking points or burst the heart and lungs, or the solution might spurt out from the openings of the animal. 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 (Fig. 3j).

31 From this point, refresh 'the pumping reference tube slot' every day. To do this, note that the reference tube has two slots for pumping. The pumping can work on either one of the two slots (Fig. 3k, top).

▲ CRITICAL STEP After many hours of pumping at high pressure, the running slot can get deformed by the movement of the gearwheel of the pump, thus reducing the pumping. Therefore, alternate the slots every 8–16 h (Fig. 3k, middle and bottom and Supplementary Video 3).

32 After washing with PBS, the perfusion should continue with the next solution. In general, the exchange of the perfusion solution is performed without touching the perfusion setup or the animal: first, stop the pumping, then completely suck out the PBS with a 50 ml serological pipette (Fig. 3l).

▲ CRITICAL STEP This operation must be done by avoiding the formation of bubbles inside the perfusion tube. To this end, do not accidentally touch the sucking tube, and make sure that it is well pushed into the chamber with the sucking end touching the bottom of the chamber. In this way after removing the PBS, some remaining PBS with its surface tension will prevent the formation of air bubbles (Fig. 3e, bottom).

33 Pour the decolorization solution inside the glass chamber, covering the animal. Start the pumping with the same pressure optimized in the previous step and perfuse for 2 d at RT. In between, exchange the decolorization solution whenever it turns into a strong yellow color (every ~12 h, Fig. 4a), indicating that the heme is being successfully eluted out from the body. On the last exchange, the solution will stay colorless or turn 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 d of decolorization, the spleen should become pale beige color (Fig. 4b) and the body whiter.

? TROUBLESHOOTING

Decalcification

34 Following the decolorization, exchange the solution with 1× PBS and perfuse three times at RT for 2–3 h to wash the decolorization solution out from the body.

■ PAUSE POINT Animal bodies can be perfused with 1× PBS for up to 2 d.

35 After washing, exchange the PBS with the decalcification solution in the same way as described before and perfuse with the decalcification solution for 2 d at RT. The refreshing of the decalcification solution is not required.

▲ CRITICAL STEP 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

36 After decalcification, wash again by perfusing with 1× PBS three times for 2–3 h at RT.

■ PAUSE POINT Bodies can be perfused with 1× PBS for a maximum of 2 d.

Permeabilization and staining

37 Replace the PBS with the permeabilization solution and perfuse with the permeabilization solution at RT for half a day.

38 After permeabilization, turn off the pump, take a 0.22 μm filter and wet it with some permeabilization solution using a syringe to reduce the formation of bubbles later in the pumping tube, Fig. 4c. Then take the sucking (inflow) end of the perfusion tube and connect it to the filter (Fig. 4d). Finally place the tube filter inside the chamber (Fig. 4e).

▲ CRITICAL STEP The filter prevents the accumulation of dye aggregates into the sample by filtering the staining solution. Periodically check if the filter still functions. If clogging is observed after extended perfusion, replace the filter.

? TROUBLESHOOTING

39 With the pump still turned off, replace the old permeabilization solution with fresh permeabilization solution (Fig. 3l). From now on, this fresh permeabilization solution will be called staining solution since it will contain the dyes.
▲ **CRITICAL STEP** At this point, if the liquid superglue was already applied as indicated in the optional point of the sample and perfusion system setup section, Step 28, and the glues still holds well, proceed with covering the whole animal with 250 ml of staining solution. If the glue was not applied yet, initially pour an amount of staining solution that does not cover the heart and apply the super glue by following the procedure described in Step 28: briefly add one to two drops of superglue onto the point where the needle goes into the heart, let the glue dry and add staining solution to completely immerse the animal. Last, make an incision in the right ventricle (Fig. 3i,j).

40 Add the dyes into the staining solution (Fig. 4f) with a pipette. For nanobody concentrations, see Table 1. Optionally, perform counterstain of nuclei by adding a nucleus dye: for example, add 290 µl of PI from the stock bottle into the staining solution.
▲ **CRITICAL STEP** The nanobody must be previously validated in sections.

41 Cover the chamber with aluminum foil to minimize light exposure (Fig. 4g).

42 Turn on the pump and perfuse the animal for at least 6 d with the staining solution. During this step place the infrared lamp at a distance of 20–30 cm from the chamber and direct the infrared light to it (Fig. 4g). The infrared light will heat up the solution to ~28–30 °C, to increase the molecular movement of the dyes for a better staining. Alternatively, place the pump and the whole setting in a temperature controlled warm room (~28–30 °C) or onto a hot plate. The temperature of the hot plate should be adjusted in order to warm up the solution in the chamber to ~28–30 °C. If the PI is added, different organs of the body such as intestine, lymph nodes and thymus will turn pink (Fig. 4h).
! **CAUTION** The infrared lamp can overheat after continuous usage for many hours. Switch the lamp off after 10–12 h to let it cool down.
▲ **CRITICAL STEP** During daily checks, if the level of the solution in the chamber decreases due to evaporation, fill it back with distilled water only.
? TROUBLESHOOTING

43 After 6 d, remove the perfusion tube with the needle from the heart, by delicately detaching the glue. Place the body in a 50 ml tube or in a bigger plastic container with a lid. Fill the tube or the container with fresh staining solution and add 5 µl of nanobody (2.5–5 µg in the 50 ml tube) (Fig. 4i).

44 Passively incubate the tube at 37 °C or at RT for 1–2 d 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 RT incubation for nanobodies that would normally require passive mild-vDISCO for the staining of dissected organs.

45 Meanwhile, discard the filter and the solution left in the glass chamber. Wash the now empty container and the pump tubing by pumping two to three times for 30 min with distilled water and then with PBS using the same pumping setup.

46 After 1–2 d, 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 two times at RT for 2–3 h. From this step, the filter is not needed anymore.

47 In the end, wash the body by perfusing it with 1× PBS two times at RT for 3 h.
■ **PAUSE POINT** Stained samples can be stored in PBS for up to 1 d at 4 °C protected from light.

? TROUBLESHOOTING

Clearing ● Timing 1–4 d

48 After staining, place the samples immersed in 1× PBS into containers resistant to organic solvents (tubes for small samples and glass chambers for whole bodies as shown in Fig. 4k) that will be used for clearing.

49 Remove the PBS and dehydrate and delipidate the samples by incubating with a series of THF dilutions (50%, 70%, 80% and two times 100%) and 100% DCM with gentle shaking, at RT, with the container lids sealed with parafilm and protected from light (Fig. 4k). Tables 2 and 4 include the incubation times and the dilutions of the organic solvents based on the samples size. For samples in small tubes (e.g., 5 ml tubes), the exchange of the solutions can be performed with a plastic Pasteur

pipette. For samples in bigger containers (e.g., 50 ml tubes or glass chambers), the exchange is done by directly pouring the previous solution into a big beaker for waste, and adding the next solution into the container. The amount of the clearing solution at each step must almost fill the plastic tube (e.g., 4–4.5 ml into 5 ml tubes) or cover the whole body in the glass chamber (~200–250 ml).

▲ **CRITICAL STEP** 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 contamination.

50 Exchange the DCM with the RI matching solution BABB. The sample must be completely immersed. Incubate for 20 min to 12 h with gentle shaking, at RT and protected from light, until the sample becomes visually transparent (Fig. 4l).

■ **PAUSE POINT** Store the cleared samples in BABB at RT and protected from light. Unimaged whole bodies can be kept for some weeks in the glass chambers used for clearing. However, for long-term storage, the bodies should be moved into plastic containers filled with BABB (e.g., 50 ml tubes). Small samples can be stored in BABB in the same tubes used for clearing.

▲ **CRITICAL STEP** Label the tubes with permanent pens and cover the text with transparent sticky tape to protect the label from solvents.

? TROUBLESHOOTING

51 *Imaging*. Imaging options should be chosen on the basis of the desired application:

2D epifluorescence microscope (AxioZoom) imaging ● Timing 5–15 min

(i) Use an adequate imaging container that allows the sample to be completely immersed in BABB. For example, small samples can be placed in smaller solvent resistant transparent containers with a flat bottom (e.g., glass Petri dishes) (Extended Data Fig. 5a–c), while the whole cleared body can be placed in the same glass chamber used for clearing (Extended Data Fig. 5d).

! CAUTION Perform all the next imaging steps wearing nitrile gloves and safety goggles.

(ii) Put the container with the sample and without the lid under the epifluorescence microscope coupled with the 1× objective (Extended Data Fig. 5b–d).

(iii) Place the sample in the chamber as straight as possible and avoid accidental movements of the sample.

? TROUBLESHOOTING

(iv) Focus on a part of the sample where it is expected to detect fluorescence signal.

? TROUBLESHOOTING

(v) Adjust the zoom on the basis of the details that need to be visualized. For example, select a zoom factor ×7 for the 2D whole-body reconstruction. Normally, small cells such as microglia can be imaged with a zoom about ×63 or higher.

(vi) Start scanning by taking individual 2D images over the sample. Proceed by covering the entire sample.

▲ **CRITICAL STEP** Move the chamber in *XY* directions very slowly to prevent any accidental sample movement.

▲ **CRITICAL STEP** Consecutive 2D images must have overlapping regions at the edges of the images to facilitate the stitching.

(vii) Save each scan as a multichannel scan if doing multicolor imaging.

Light-sheet LaVision-Miltenyi Biotec Ultramicroscope II imaging ● Timing 2–12 h

(i) Mount a zoom body unit onto the microscope by following the manufacturer's instructions.

! CAUTION Perform all the next imaging steps wearing nitrile gloves and safety goggles.

(ii) Pour BABB into 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 manufacturer (Extended Data Fig. 6a) or plunged onto Micro-Fine Ultra needles attached to the sample holder[16] (Extended Data Fig. 6b–e). Bigger samples such as the chest or the abdomen can be mounted on the sample holder using the suggested superglue (which does not affect the imaging) (Extended Data Fig. 6f–j). For using superglue: attach a piece of black sticky tape onto the surface of the sample holder (Extended Data Fig. 6g), apply one drop of Maxi-Cure super glue onto the tape (Extended Data Fig. 6h), inject 30–40 µl of the Insta-Set Accelerator over the super glue using a 1 ml syringe + needle (Extended Data Fig. 6i), lightly wipe the sample two to three times on a paper tissue (e.g., Kleenex) to remove some BABB, place the sample onto the glue and hold it for 1 min until it is

stabilized (Extended Data Fig. 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 STEP** Mounting of the samples should be carried out as fast as possible because air can go into the tissue that it is not soaked in BABB, creating air bubbles. Be gentle while grabbing the samples with tweezers and do not squeeze them to avoid the accumulation of air bubbles inside. For the removal of bubbles, see Extended Data Fig. 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, which can damage the mechanical components of the microscope.

(vi) Select the correct filter set for the fluorophore in the software.

(vii) Turn on the excitation light and move the sample in the *Z* direction until the upper surface of the sample is illuminated by the light sheet.

(viii) Mount the objective of interest. We use the 4× immersion objective for the scan of organs that can fit in a tiling scan of maximum 3 × 4 (e.g., samples smaller or equal to a whole brain), the 2× immersion objective for other body parts (e.g., limbs, chest), the 12× and the 20× immersion objectives to detect very small structures such as single cancer cells, dendritic spines or 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 the solution does not overflow; otherwise, use a Pasteur pipette to suck out some BABB from the imaging chamber. Water immersion objectives such as Zeiss 20× can be compatible with BABB, but in general, the compatibility of objectives must be checked in advance with the manufacturer or with preliminary tests.

(x) Move the objective slightly up and down to adjust the focus.

? TROUBLESHOOTING

(xi) Adjust the microscope settings on the basis of the sample. For example, for a Thy1-GFPM brain we used a *Z*-step of 4 µm, double-sided light sheet, NA 0.025, light-sheet width 60%, exposure time 100.00 ms, tile scan 3 × 3 with 13% of overlap and 5,000 µm of *Z*-range.

▲ **CRITICAL STEP** The laser power and the exposure time should be optimized to never reach the saturation of the camera.

? TROUBLESHOOTING

(xii) Adjust the alignment of the two 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, when performing multichannel imaging.

(xiv) Select two-sided light sheet or one-sided light sheet on the basis of the size of the sample. For small or thin samples such as a spinal cord, one-sided light sheet is sufficient to cover the entire sample.

(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.

Light-sheet imaging with the LaVision-Miltenyi Biotec Blaze microscope ● Timing 6–7 d

(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 Extended Data Fig. 6k–o. In case of a whole body, you can start mounting the body either 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 (Extended Data Fig. 6k, l). Suck 50–70 µl of the Insta-Set Accelerator solution with a 1 ml syringe and keep it for later. Apply three to five drops of Maxi-Cure super glue onto both pieces of the tape (Extended Data Fig. 6m). After that, inject ~20–30 µl of the Insta-Set Accelerator into each drop of the glue (Extended Data Fig. 6n) in a fast manner. Place the body onto the sample holder and hold it for few minutes until the glue cures

(Extended Data Fig. 6o). The removal of the glue after the scan will be easily done by detaching the tape.

▲ **CRITICAL STEP** Avoid leaving the sample outside of BABB for too long, and perform the sample mounting fast. Otherwise, air can go into the sample and create air bubbles within the tissue. Be gentle while grabbing the samples with tweezers and do not squeeze them to avoid the accumulation of air bubbles inside the sample. For the removal of air bubbles, see Extended Data Fig. 6p–r.

? TROUBLESHOOTING

(iii) Follow steps (iv)–(vii) of the 'Light-sheet LaVision-Miltenyi Biotec Ultramicroscope II imaging' section.

(iv) Select the objective of interest. We use the 1.1× objective to image whole bodies, and the 12× objective to capture details.

(v) 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 the solution does not overflow; otherwise, use a Pasteur pipette to suck out some BABB from the imaging chamber.

(vi) Move the objective slightly up and down to adjust the focus.

? TROUBLESHOOTING

(vii) Adjust the microscope settings. For example, to cover a whole adult mouse, set 3 × 8 tiles of scans with 35% of overlap, two-sided light sheet, laser power 10–15%, *Z*-step 6 µm, light-sheet NA 0.035, light-sheet width 80–100%, exposure time 80.0–120.0 ms and 11 mm of *Z*-range. Settings can be adjusted on the basis of the characteristics of the sample.

▲ **CRITICAL STEP** The laser power and the exposure time should be optimized to never reach the saturation of the camera, while the number of tiles should be set on the basis of the sample size.

? TROUBLESHOOTING

(viii) Adjust the laser power and the chromatic correction focus for the other channels, if performing multichannel imaging. Start the scanning.

(ix) 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.

(x) Flip the body or sample and mount it onto the sample holder with the super glue + accelerator method indicated before.

▲ **CRITICAL STEP** When in prone position, the animal might appear tilted in *Z* because the thickness of the head of the animal including the snout is higher than the thickness of the belly.

(xi) Scan the other side in the same way as described in steps (iii)–(ix) of this section.

Light-sheet imaging with a modified mesoSPIM ● Timing 10 h to 2 d

(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) (Extended Data Fig. 7d).

(iv) Attach the lid of the sample cuvette (Extended Data Fig. 7c).

(v) Insert the sample into the microscope by attaching the lid magnet to the rotation stage (Extended Data Fig. 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 STEP** 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) Test the range of motion for the intended scan by moving the sample around to ensure that the sample and the sample holder will not collide with the imaging chamber during the scan and tiling.

(viii) Before starting the 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, the 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 software 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 12,000 µm and 20,000 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) 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) Use the preview button in the acquisition manager to check whether individual tiles in the acquisition manager are set up properly.
▲ **CRITICAL STEP** 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. The mesoSPIM-control software 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, rotate the sample by 90° or 180° to perform a multiview acquisition.

Lightsheet Z.1 microscope imaging ● Timing 10–30 min

(i) Switch on the microscope, start the ZEN software and mount the objectives required for the image acquisition.

(ii) Glue the cleared sample to the Lightsheet Z.1 sample holder (Extended Data 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.

(iii) Fill the chamber with the clearing solution (e.g., BABB) and insert it into the microscope.

(iv) Lower the sample and position it in front of the detection objective, rotate the sample to the desired angle (Extended Data 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.

(v) 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 (Extended Data Fig. 8c).

(vi) Set up tracks for imaging by adjusting the laser intensity, exposure time, zoom and activate the Pivot scanner to maximally reduce the stripe artefacts in the images. Use simultaneous two channel imaging to reduce the total time of the experiment.

(vii) Define the *Z*-stack and the Tile scan for the entire brain.

(viii) Start the image acquisition.

(ix) Save the data for further processing.

Inverted confocal microscope imaging ● Timing 1–3 h

(i) Cut the sample and place it onto the glass slide of a glass bottom dish (Extended Data Fig. 5e, f).

(ii) Cover the sample with a few drops of BABB (Extended Data Fig. 5e, f). Complete immersion of the sample in BABB is not necessary, as long as the interface between the sample and the glass has BABB. Closing or sealing of the Petri dish is not required, because BABB has a high evaporation point and does not dry out for several days.

(iii) Mount the glass-bottom dish onto the sample holder of the microscope (Extended Data Fig. 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 h of imaging without leaking. However, BABB will eventually melt the plastic after some time. The resistance of the glass bottom dishes with BABB should be tested before mounting them onto the microscope. Strictly avoid leakage or spillage 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-WD objective such as the 25× Leica (used here) can be used 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, Z-stack limits, etc.

(vi) Activate the online stitching.

(vii) Start the scan.

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

52 Image processing and visualization steps will depend on the microscope method used:

Processing of 2D epifluorescence microscope (AxioZoom) scans ● Timing 30 min to 1 h

(i) From each scan, export the single 2D image.

(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 their opacity whenever merging the overlapping regions.

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

(vi) Save the stitched file in Photoshop format such as.PDD, *.PSD. Then the stitched image can be exported as .jpeg or .tiff file and loaded in Fiji for visualization, analysis and the separation of the individual channels.

Processing of light-sheet microscope (Miltenyi Biotec) scans ● Timing 2 h to 6 d

(i) The Miltenyi Biotec light-sheet microscopes (Step 51B,C) save the scans as series of TIFF files in separate/not stitched sequences in one folder. 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 Fig. 1a).

(ii) Fill the 'Stitch Image Grid Sequence' with the information about grid size, overlap, input directory, output directory and start positions (Supplementary Fig. 1b).

(iii) In 'file names': copy the name of one of the images from the scan and replace [00 ×00] with [{yy} x {xx}] and Z0000 with Z{zzzz}.

(iv) 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, 'start z' = a number of an optical slice deep in the tissue that contains data from each tile. This will yield the correct parameters for all tiles.

(v) If the stitched result looks fine, generate the first stitched image by unclicking 'create only preview' and start stitching.
▲ CRITICAL STEP If there are mistakes in *XY* dimensions, manually correct the positioning of the tiles in 2D, by using the TrakEM2 plugin of Fiji.
▲ CRITICAL STEP Stitching for all sections can be performed with this plugin by simply changing '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 steps (vi)–(vii).

(vi) Immediately after starting the stitching, the 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 Fig. 1c). 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 it into a new folder for stitching another channel. Each channel requires its own .txt file with the same coordinates; only C0x should change (Supplementary Fig. 1c, d).

(vii) In Fiji, open the macro for stitching called 'Stitching-old-just_txtFile.py' (available in http://discotechnologies.org/) and click on 'Run'. Load the .txt file that was just created in the previous step, which contains the stitching parameters for each channel and input the 'Number of images', which is the total number of optical slices of the scan (Supplementary Fig. 1e).

(viii) Click 'OK' and start stitching the first channel. Conduct the same steps for each channel.
▲ **CRITICAL STEP** Stitching can also be performed by other algorithms: for example, using TeraStitcher[62] (v.1.10; https://abria.github.io/TeraStitcher/), especially for correction of the shifting of the tiles in the *XYZ* directions.

(ix) Now, to stitch different tiling scans already individually stitched previously (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 Figs. 2 and 3).

(x) 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 four digits (e.g., for the channel C00, new name of the files: C00-Z0000.tif, C00-Z0001 and so on as shown in Supplementary Fig. 2a).

(xi) Save all the renamed images from different channels into the same folder (i.e., C00-C01-C02) (Supplementary Fig. 2a, last panel).

(xii) 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 Fig. 2b).
▲ **CRITICAL STEP** 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'.

(xiii) Select 'custom import'. Select the output folder and give a name to the file (e.g. GFPM34-dorsal.sis). 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 Fig. 2b).
▲ **CRITICAL STEP** All the images must be in the same format (for example, either compressed or not compressed, 16-bit and same number of *Z*-panels).

(xiv) A new window called 'manual import map' will pop up. Go to 'Selection' and 'pattern matching', to check whether Arivis identifies which part of the image name contains the information about the channel and the *Z*-panel, respectively (Supplementary Fig. 2b).

(xv) Check if the output name is correct and confirm the setting.

(xvi) Start the conversion: Arivis/Arivis converter will create a .sis file of your scan.
▲ **CRITICAL STEP** This .sis file must be saved in a final folder that 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.

(xvii) 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 the same structure for all images and follow the steps (xii)–(xvi) as before except for 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 a 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 STEP** Ensure that the second .sis file and all the others 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.

(xviii) Set the correct pixel size in μm for each volume by going to '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.

(xix) In 'Extra' → 'Preferences', select the desired quality of rendering. If dealing with large datasets (in the range of 800–900 GB), reducing the quality of rendering helps increase the speed.

(xx) Adjust the brightness and contrast and scroll in 2D to take a better look of the data

(xxi) If necessary, flip one of the volumes to match the orientation of another volume, by clicking 'Data' → 'Transformation Gallery' → 'Flipping'. In 'Flipping Properties' you can choose to flip the volume around a 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 d depending on the data size. After checking that the flipping has worked correctly in the multiview window, close the original version that was not flipped.

▲ **CRITICAL STEP** Create a .docx/.doc or a .txt file to keep track of how the volumes were flipped 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.

(xxii) Using the 2D visualization modality, look for three 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. The purpose is to get the coordinates of this landmark/pixel.

▲ **CRITICAL STEP** 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 ease the finding of the same landmark in different volumes. We generally use bones and not internal organs as landmarks.

(xxiii) To highlight the selected landmark of interest, click on the icon 'Place New Object/Marker' and click on the icon 'Marker', then, click on the landmark of interest for each image set. Then, match the names of the corresponding landmarks through 'Show objects Table' (Supplementary Fig. 2c).

(xxiv) 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 reuse them in the future). You can rename each landmark appearing in all volumes using the same name (e.g., LM1). Save after each step.

(xxv) Click on 'Data' → 'Volume Fusion'. A new prompter will open; indicate which volume you want to specify as 'Base image Set' and which 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. This will transform the whole 'Moving Image Set' 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 Fig. 3a).

(xxvi) Choose 'New File' to save the fused volume as that will be generated 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 (Supplementary Fig. 3a).

(xxvii) In 'Transformation' → 'Landmark Registration' window, click 'Add all annotations as Landmarks' for both 'Base Image Set' and 'Moving Image Set'. By doing so, the list of landmarks will appear as two lists in this window.

▲ **CRITICAL STEP** Make sure that the order of landmarks is the same in both image sets. Their locations are displayed in the volumes on the right side of the window (Supplementary Fig. 3a).

(xxviii) Click run to automatically fuse the two volumes/image sets. This step will take several minutes depending on the power of the computer.

(xxix) 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.

(xxx) 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

(xxxi) Set the scale to '100%'. Click run. This process will take 1–2 d and will create a .sis file consisting of the volumes now stitched together (Supplementary Fig. 3b).

(xxxii) 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 the data.

▲ **CRITICAL STEP** 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 needed for further analysis.

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, DeepMACT and NeuroGPS-Tree.

Troubleshooting

Troubleshooting advice can be found in Table 5.

Table 5 | Troubleshooting table

Step	Problem	Potential cause	Suggested solution
2, 3	The liver does not turn partially or completely yellow	The angle with which the PBS goes inside the heart from the needle is not optimal	Change the position or the angle of the needle Make sure the right atrium is not covered or clogged
	The animal does not become rigid and stiff with PFA perfusion	The angle with which the PFA goes inside the heart from the needle is not optimal	Change position or the angle of the needle
27	The needle does not go into the hole	The tip of the needle might be too large or the hole too small	Change the angle of the needle Slightly enlarge the hole with a larger normal needle until the size is sufficiently big for the perfusion needle
28	The glue detaches at some point during perfusion	A movement caused displacement	Glue it again
33	The decolorization solution is still yellow at the end of this step	More heme has to be extracted	Repeat one or more round of decolorization solution
35	When pumping the decalcification solution, the solution turns from transparent to milky	Insufficient wash of the decolorization solution from the previous step	Wash the sample again by pumping with PBS and restart the decalcification step
38	Presence of bubbles in the perfusion tubing	While connecting the filter to the perfusion tubing, air bubbles entered the tube	Temporarily displace the ending of the tube that holds the needle in order to remove the needle from the heart. Switch on the pump for some minutes to push all the bubbles out from the tubing through the needle. When all the bubbles are removed, place the needle back into the hole on the heart. Pump for some minutes, ensuring that the system is still working properly, and switch off the pump again for the next step
42	The perfusion system starts pumping a lot of bubbles	The filter might be obstructed for example by hair	Change the filter with a new one and repeat the operation of removing the bubbles from the tubing when the new filter is connected to the tube
47	The organs are too widespread	The abdominal cavity may have been opened too much during perfusion and fixation step	Push the organs back inside the abdominal cavity and if necessary, use surgical sutures to stitch up the body with a couple of stitches before clearing
50	The sample is blurry or not sufficiently transparent	Insufficient clearing	Increase the incubation time and/or volume of the clearing solutions and the refractive index matching solution
51A(iii)	Sample moves during epifluorescence imaging	Sample is not adequately fixed in the glass chamber	Try to put the lid of the glass chamber diagonally inside the chamber as a wall to restrain the sample
51A(iv)	No signal visible	Animal does not express the fluorescence signal in some regions or at all	Check the genotype of the animal and make sure there were no mistakes Choose another body region to image
51B(iii) and C(ii)	Presence of bubbles in the sample	Air trapped inside the sample	Gently tap the sample inside the imaging chamber with tweezers to push the bubbles out Use a syringe with a fine needle to suck the bubbles out (if you decide to inject the bubbles with BABB, sprays of BABB can reach your eyes! Wear protection) Wait for some hours until bubbles will disappear by themselves. This might take even some days; therefore, you can place the sample back into BABB into the storing container while doing this. Mount the sample again back to the microscope

Table continued

Table 5 (continued)

Step	Problem	Potential cause	Suggested solution
51B(x) and C(vi)	Sample not visible during the imaging	Out of focus	Move the stage in *X*–*Y* to find bright halos that might be the sample. Move the objective to the highest level and start lowering again very slowly while continuously adjusting the contrast on the computer screen until focus is reached
	Distortion in the image during imaging	Presence of a bubble between the sample and the objective	Move the objective up out of the BABB, wipe the lens of the objective once with a piece of tissue to get rid of the bubble. Lower the objective back into the BABB
51B(xi) and C(vii)	Tiles in mosaic scan do not seem to have proper overlap	The sample moved during the imaging	Remount the sample more firmly by, for example, increasing the glue amount
52B(xxx)	Result of stitching is not satisfactory	Landmarks that were chosen were not optimal	Replace the landmarks with new ones, by observing the preview and identifying the region that shows problems in the fusion, then change the landmark that was inside this region

Timing

Steps 1–3, sample preparation with PBS and PFA perfusion: 14 h to 1 d
Steps 4–47, passive-vDISCO: 2–23 d, whole-body active vDISCO: 12–16 d
Steps 48–50, clearing, 1–4 d
Step 51, imaging:
Option A, epifluorescence imaging: 5–15 min
Option B, light-sheet imaging with Ultramicroscope II: 2–12 h
Option C, light-sheet imaging with Blaze: 6–7 d
Option D, light-sheet imaging with the modified mesoSPIM: 10 h to 2 d
Option E, light-sheet imaging with Z.1: 10–30 min
Option C, confocal imaging: 1–3 h
Step 52: image processing:
Option A, processing of 2D epifluorescence microscope (AxioZoom) scans: 30 min to 1 h
Option B, processing of light-sheet microscope (Miltenyi Biotec) scans: 2 h to 6 d

Anticipated results

By developing a reproducible, straightforward and rapid pipeline that combines different steps that have the purpose to decrease the tissue background, increase and stabilize the fluorescent signal and clear very large specimens, we are able to obtain high-resolution 3D imaging data of an entire rodent.

Previously, one of the applications of vDISCO was to detect and localize all metastases at single-cell resolution in animal models of cancer: the data shown in Fig. 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 (but are not visible in bioluminescence imaging in Fig. 2a,b). This kind of data can be analyzed using artificial intelligence algorithms that are able to provide the location, number and distribution of all cancer cells and their targeting by therapeutic antibodies[1].

Here, we also show the possibility of analyzing other biological structures that span from head to toe: Fig. 8 represents the 3D reconstruction of the *Prox1*+ lymphatic system where details of lymphatic vessels are visible in different organs (Supplementary Video 4). Such data can become highly valuable for studies of whole-body inflammatory responses from local diseases. For example, on the one hand our light-sheet data reveal the intricate lymphatic vasculature in the lungs (Fig. 8i, red arrows); on the other hand, our confocal data magnify a single connected lymphatic vessel (Fig. 8j). It has been shown that patients with moderate chronic obstructive pulmonary disease bear de novo lymphatic vessel formation in lungs[71]. vDISCO can help systematically assess changes (e.g., connectivity, diameter and length of the vessels) in the lymphatic vasculature of the disease mouse model not only in the organ affected by the disease but also in other peripheral organs. In addition to the widely known presence of *Prox1* expression in lymphatic vessels[72], our technology underlines the less known cells where *Prox1* is also expressed, such as the satellite cells in muscle tissue (Fig. 8g,k, green arrows)[73] and granule cells in the hippocampus[74]. On the basis of this unbiased screening, it is

possible to analyze the different patterns of expressions and how this pattern may change under specific circumstances.

Thus, vDISCO can be applied in synergy with many different fluorescent imaging systems, including epifluorescence microscopes, confocal microscopes and different kinds of light-sheet microscopes (Figs. 2 and 5–9 and Extended Data Figs. 2, 3 and 5–8).

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

Last, we show that vDISCO can be used to trace viral spread in tissue: in Fig. 9a–c, the increase and stabilization of the fluorescent signal expressed by viral vectors after vDISCO allows us to detect neurites including single axons (Fig. 9b,c, red and cyan arrowheads, and Supplementary Video 5).

Overall, vDISCO can be broadly applied to various studies. We think vDISCO may be used in future work to elucidate how peripheral symptoms originate from brain insults or to investigate alterations in the neuronal circuitry in neurodegenerative and psychiatric diseases. Other possible future applications include the study of whole-body inflammatory phenomena, the crosstalk between microbiota and host, and the screening of infectious agents, stem cells or therapeutic cells (e.g., CAR-T cells). The ability to comprehensively and unbiasedly analyze whole organs and bodies represents a tool for biomedical researchers to address a broad spectrum of scientific questions that require single-cell resolution with a systems-biology approach.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The main data discussed in this protocol are available in the supporting primary research papers (https://www.nature.com/articles/s41593-018-0301-3 and https://www.cell.com/cell/fulltext/S0092-8674(19)31269-3?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS0092867419312693%3Fshowall%3Dtrue). The raw datasets of the main data and of the additional new data shown in this work are too large to be publicly shared but are available for research purposes from the corresponding authors upon reasonable request.

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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 pericyte-labeled brains. F.F.V., M.M., T.-L.O. and F.H. provided mesoSPIM data. C.V. and J.A.V.G. provided the custom-made nanobodies anti-GFP. D.K. contributed to the imaging, and M.I.T helped with the stitching of the Prox1-EGFP sample. R.C. and Z.I.K. supervised the experiments. A.E. supervised the project. R.C. and Z.I.K. wrote the manuscript. All authors edited the manuscript.

Competing interests

A.E., R.C., C.P. and S.Z. have filed a patent related to vDISCO.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41596-022-00788-2.

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Key references using this protocol

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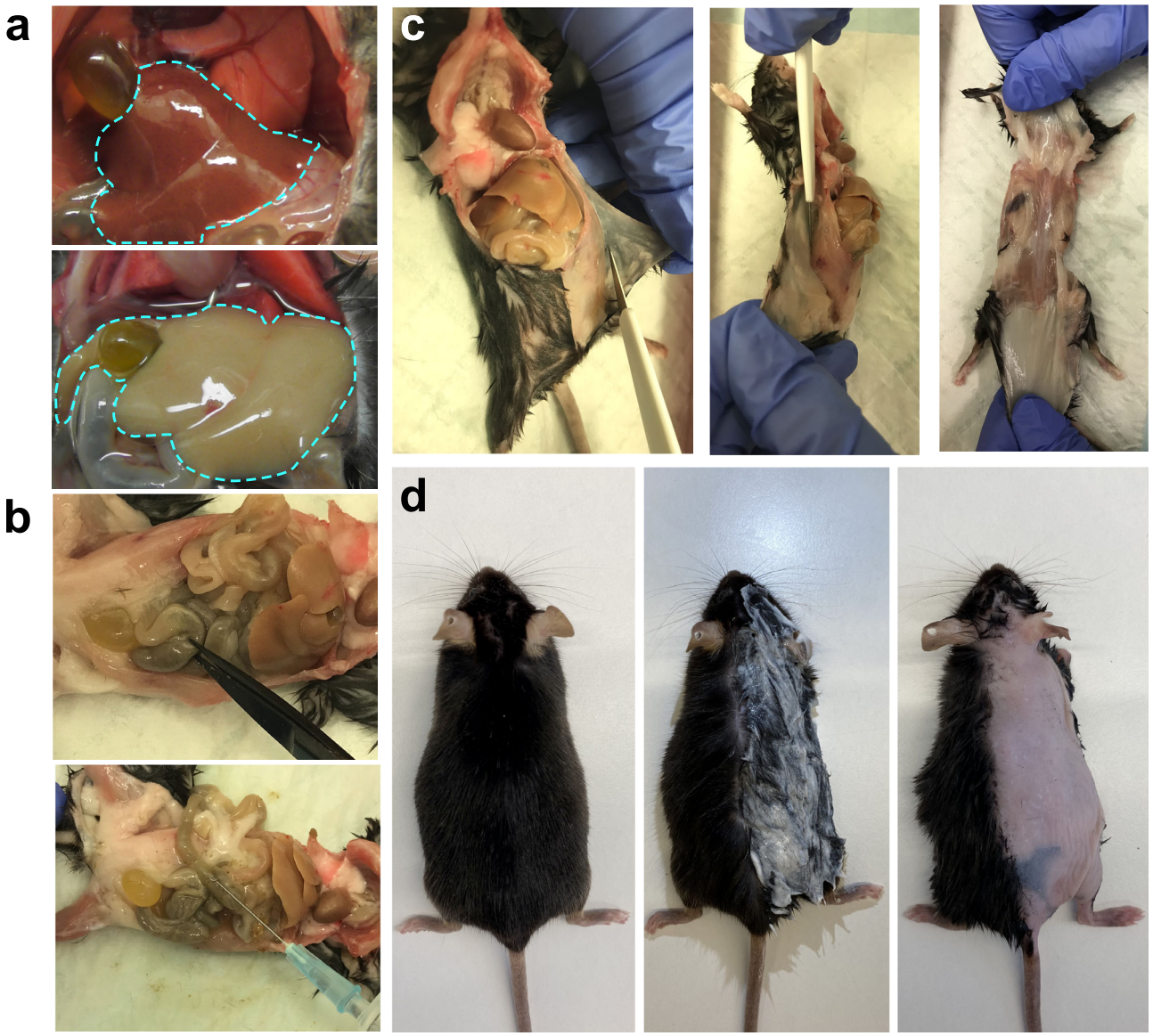
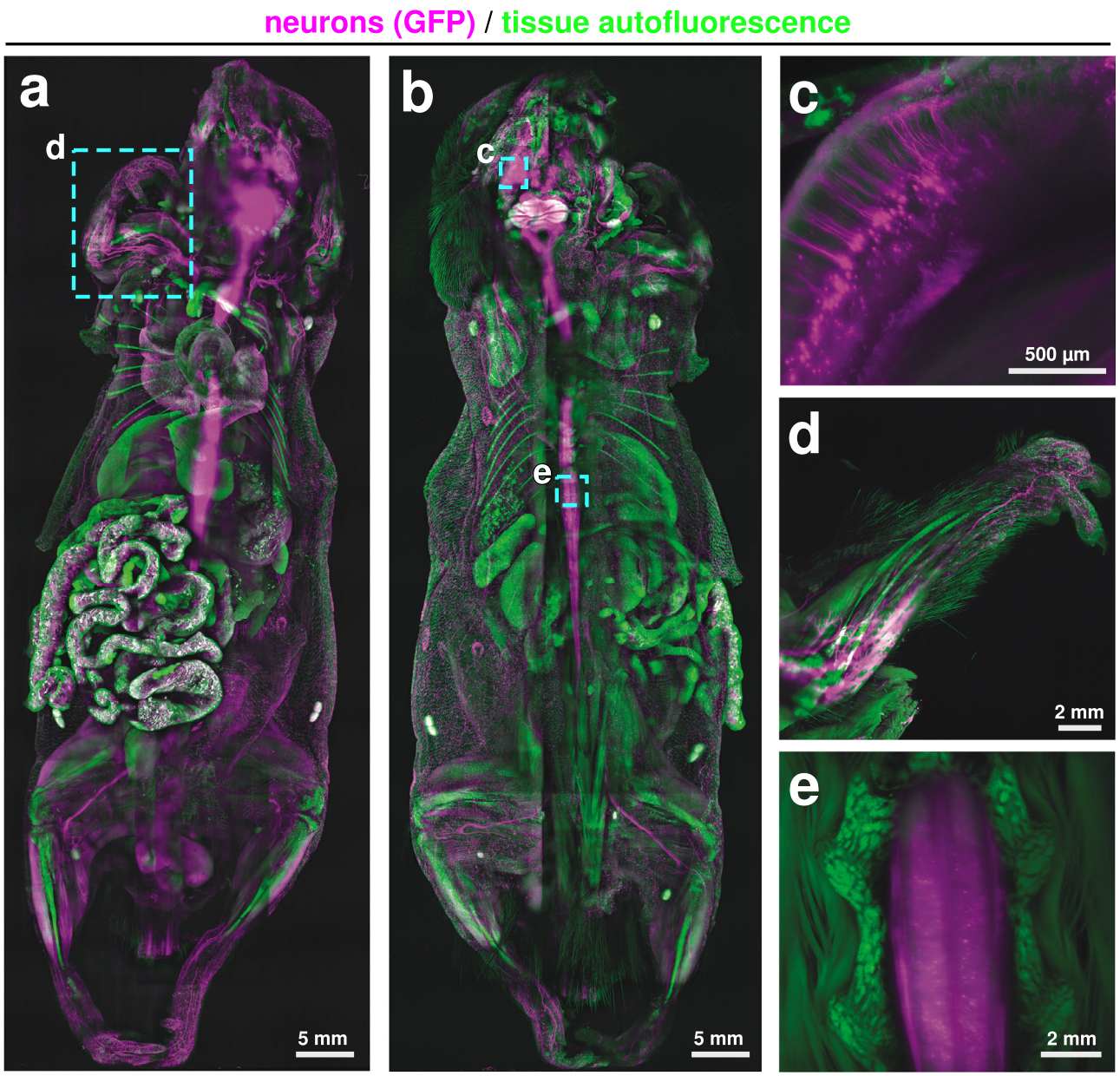
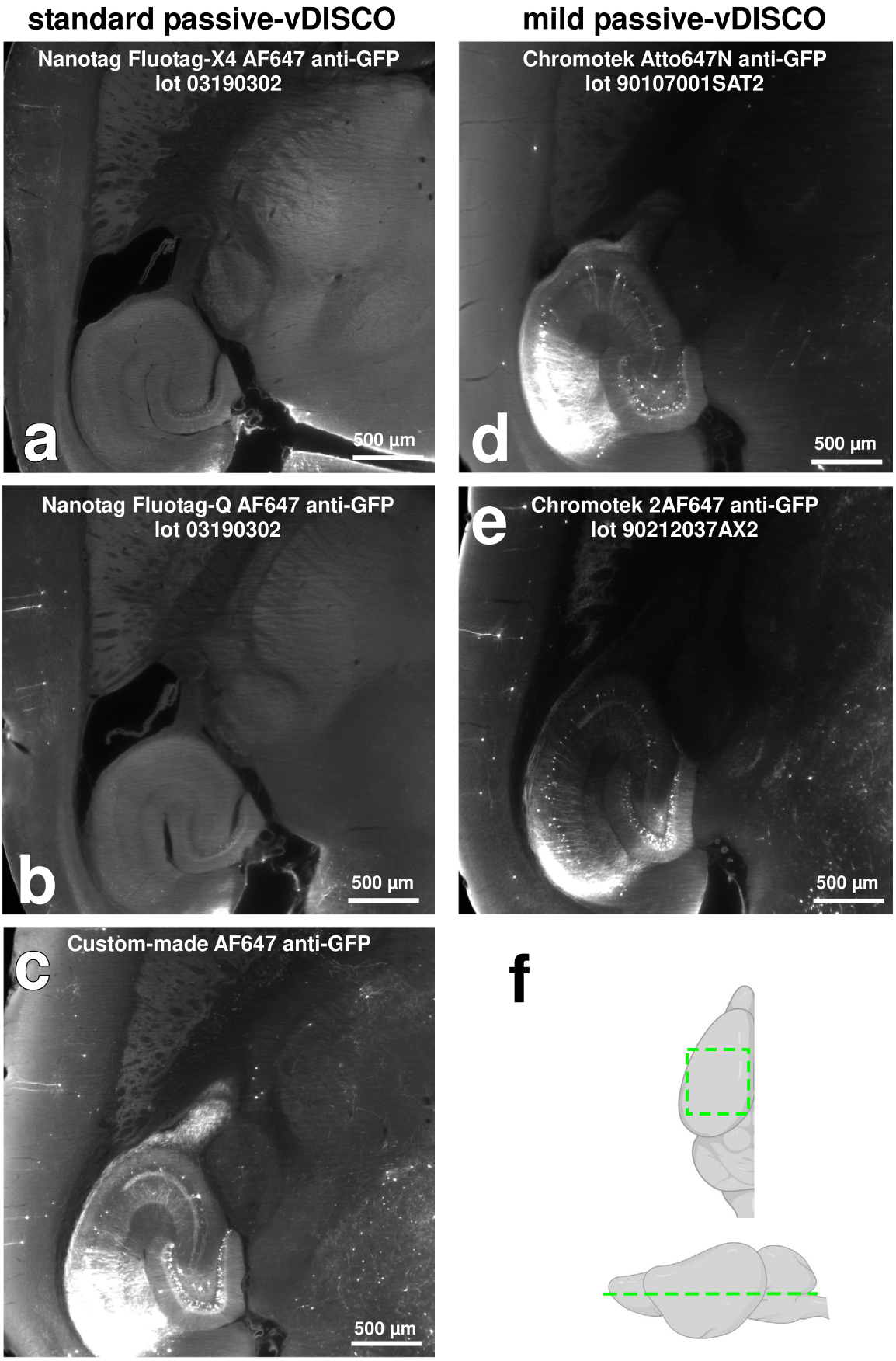


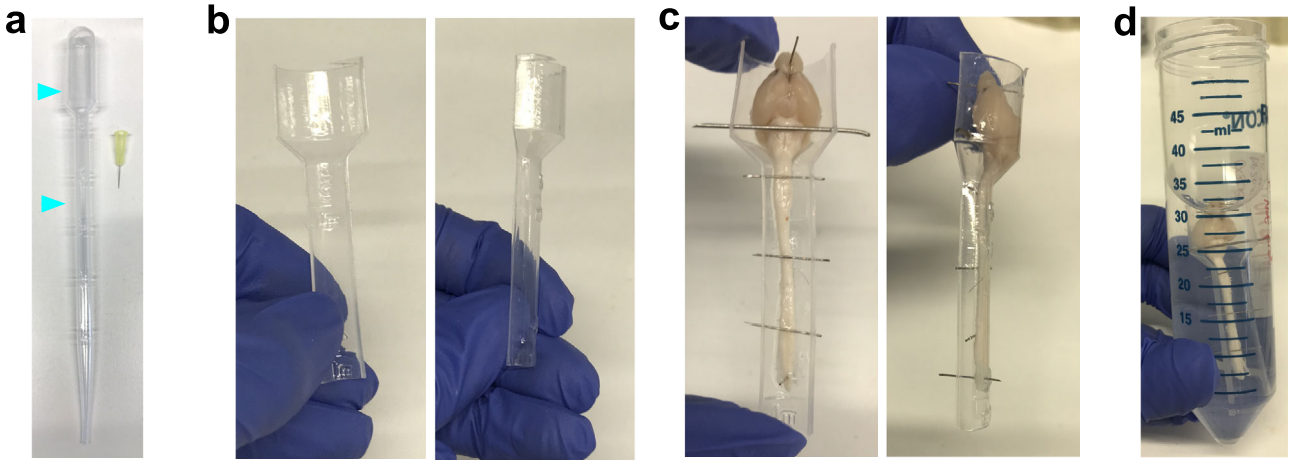
Extended Data Fig. 1 | Sample preparation for whole-body active-vDISCO. a, During the 1× PBS perfusion step of the anesthetized animal, the color of the liver (cyan dashed line) turns yellow as indication of proper drainage of the blood from the body. **b**, Some cuts in the gut are necessary to flush 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**, 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 min. Steps can be repeated until the desired quality of hair removal is achieved. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.



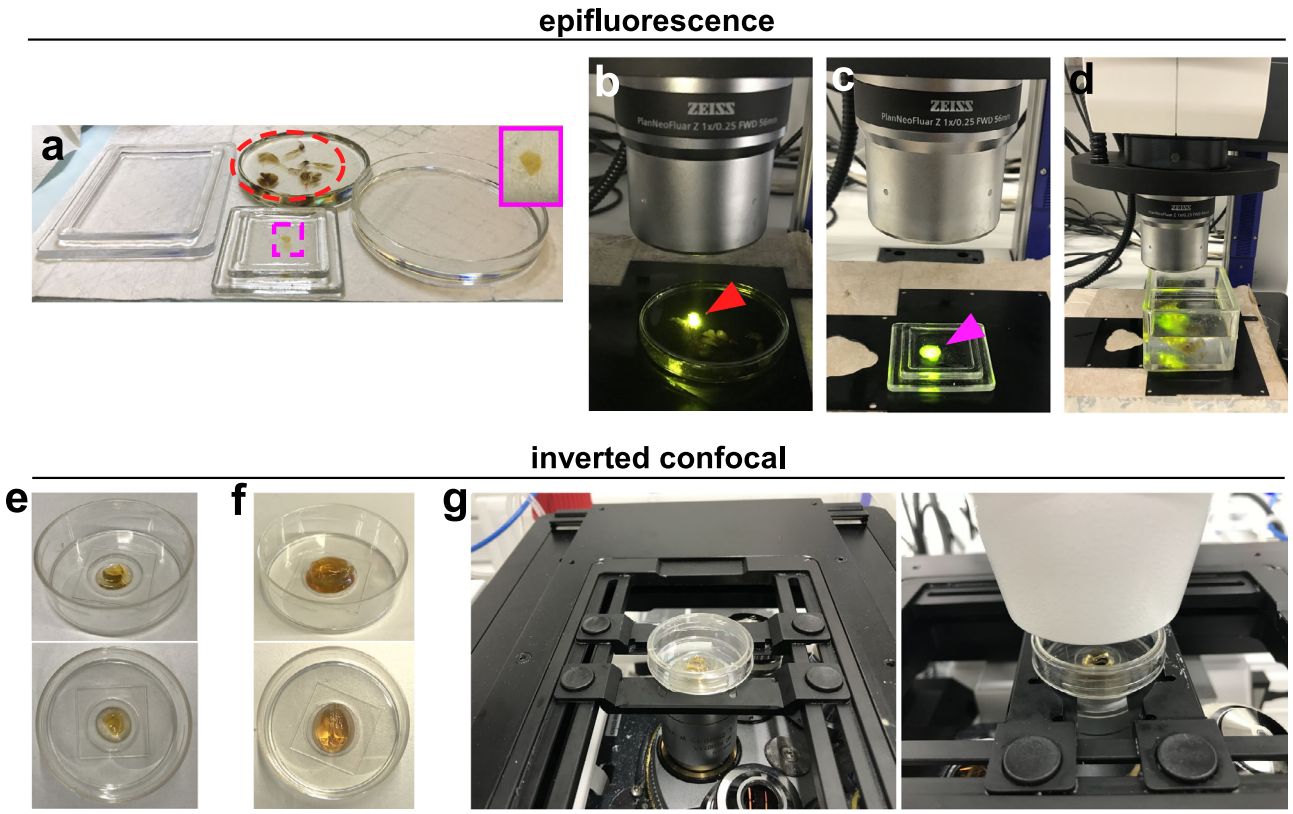
Extended Data Fig. 2 | Whole-body imaging of a sample with intact skin using a modified mesoSPIM light-sheet microscope. a, Maximum projection of an adult Thy1-GFPM mouse with intact skin imaged with a modified mesoSPIM light-sheet microscope from the ventral side. Autofluorescence excited at 488 nm 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 0.9× magnification whereas image in **c** was imaged at 4× magnification. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.


standard passive-vDISCO
mild passive-vDISCO
Nanotag Fluotag-X4 AF647 anti-GFP
lot 03190302
a
500 µm
Chromotek Atto647N anti-GFP
lot 90107001SAT2
d
500 µm
Nanotag Fluotag-Q AF647 anti-GFP
lot 03190302
b
500 µm
e
Chromotek 2AF647 anti-GFP
lot 90212037AX2
500 µm
c
Custom-made AF647 anti-GFP
500 µm
f

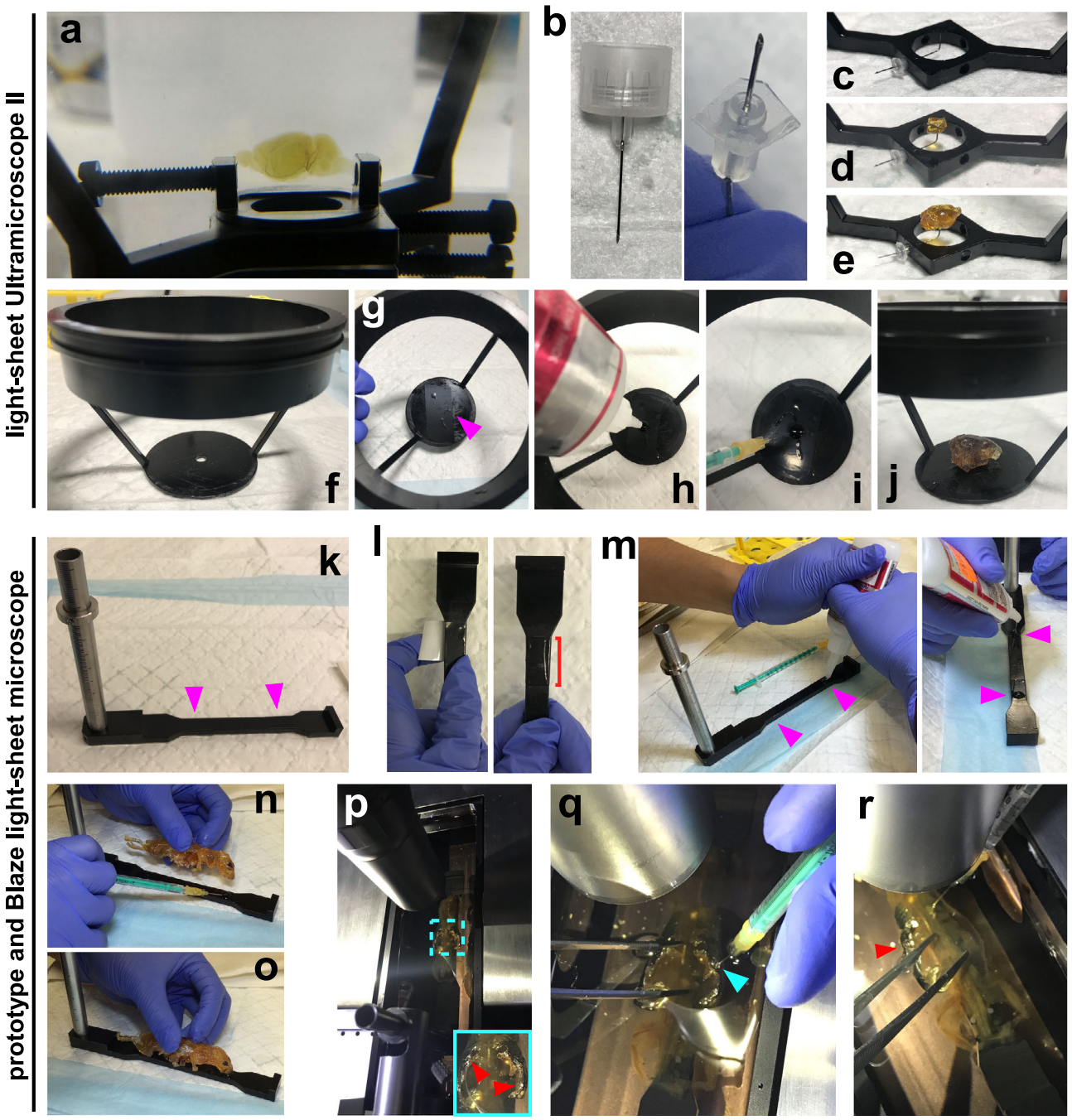
◀ **Extended Data Fig. 3 | Performances of different kinds of nanobodies in passive-vDISCO for Thy1-GFPM line. a**–**e**, Light-sheet microscopy images of half mouse brains from Thy1-GFPM lines showing the performances of different batches of nanobodies from different sources and companies using standard passive-vDISCO (**a**–**c**) and mild passive-vDISCO (**d** and **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 two independent experiments for each kind of nanobody. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.



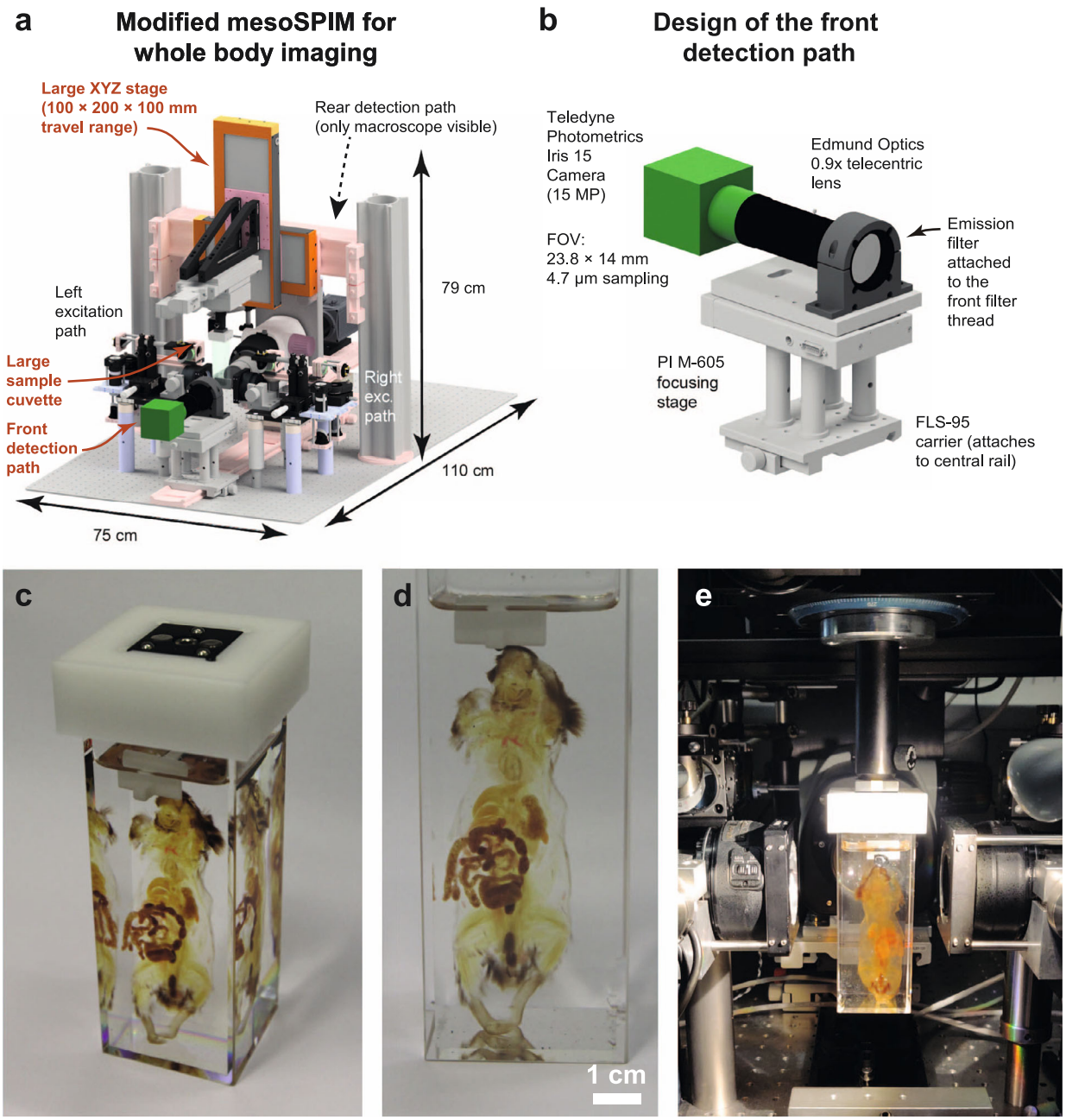
Extended Data Fig. 4 | Strategy to make spinal cord straight for passive-vDISCO. a, Required materials: a plastic Pasteur pipette and some fine needles. The cyan arrowheads indicate the cutting points. **b**, The plastic Pasteur pipette is then longitudinally cut in half. **c**, Positioning of the needles 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 50 ml tube for passive-vDISCO protocol. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.



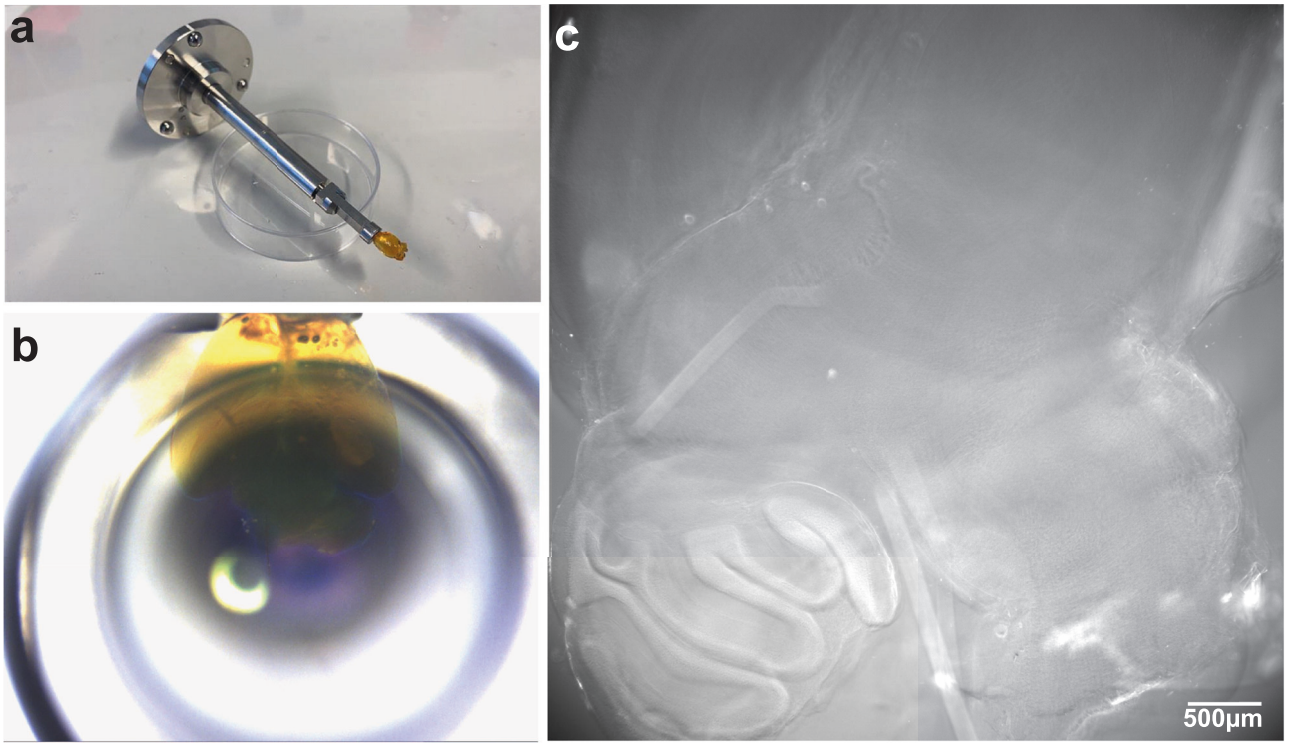
Extended Data Fig. 5 | Mounting of cleared samples for epifluorescence imaging and inverted confocal imaging. a–d, Mounting of different samples for AxioZoom epifluorescence imaging: different glass containers used to mount cleared organs (red dashed circle) and slices (magenta boxes) for AxioZoom epifluorescence imaging (**a**); epifluorescence imaging of dissected organs (red arrowhead) and slices (magenta arrowhead) with the AxioZoom microscope (**b** and **c**); epifluorescence imaging of the whole body with the AxioZoom microscope (**d**). **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**). Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.



Extended Data Fig. 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 stuck to the flat surface (**g**, magenta arrowhead), 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** and **l**, magenta arrowheads), superglue is applied onto the black tapes (**m**, magenta arrowheads), 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** and **r**, red arrowheads) from the samples (e.g., a whole body) by inserting a fine needle into the bubble (**q**, cyan arrowhead) and sucking the bubble out with the syringe (**r**). Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.



Extended Data Fig. 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 40 × 40 × 120 mm^3 quartz cuvette. The cuvette was closed with a custom lid that includes a kinematic mount with magnets that attach to the *XYZ* stages. **d**, To stabilize 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. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.



Extended Data Fig. 8 | Whole-brain imaging with Zeiss Lightsheet Z.1. a, The cleared brain sample is glued to the Lightsheet Z.1 rotatable 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. Animal experiments followed European directive 2010/63/EU for animal research, reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria, complied with the '3Rs' measure and were approved by the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and conformed to institutional guidelines of Klinikum der Universität München/Ludwig Maximilian University of Munich). The severity of the procedure was low.

nature portfolio

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Our web collection on statistics for biologists contains articles on many of the points above.

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Policy information about availability of computer code

Data collection	*Code and software versions and descriptions used to collect the data in this study are listed in the section "Data processing and analysis tools/software" of the article*
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All studies must disclose on these points even when the disclosure is negative.

Sample size	*not applicable*
Data exclusions	*Animals that were negative for the expression of the fluorescent proteins after genotyping were excluded from the study.*
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Study description	
Research sample	
Sampling strategy	
Data collection	
Timing	
Data exclusions	
Non-participation	
Randomization	

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Study description	
Research sample	

Research sample

Sampling strategy

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Timing and spatial scale

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Did the study involve field work? ☐ Yes ☐ No

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☒	☐ Dual use research of concern

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Antibodies used: *All nanobodies used in this study are shown in Table 1 with their RRID that includes all relevant information. Used nanobodies are*

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Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

Authentication

Mycoplasma contamination

Commonly misidentified lines
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Palaeontology and Archaeology

Specimen provenance

Specimen deposition

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Young mixed gender mice of the following lines are used in this study:Thy1-GFPM37 (and Thy1-Y FPH), CX 3CR1GFPI+ (B6.129 P-Cx3cr1tm1LittlJ ; Jackson Laboratory strain code 00558 228), Prox1-EGFP (Tg(Prox1-EGFP)KY 221GsatIMmucd; Mutant Mouse Resource and Research Centers strain code 031006-UCD) and PDGFRb-EGFP (Mouse Genome Informatics strain code 4847 307), C57 BLI 6 mouse transplanted with murine syngeneic R254 pancreatic cancer cells expressing eGFP for 38 days, an adult Emx1-Cre x RΦ GT. mice64 ,65 inj ected with EnvA-pseudotyped G-deleted rabies virus expressing GFP (SADB19 66) in the neocortex.

Wild animals

This study does not involve wild animals

Field-collected samples

This study does not involve Field-collected samples

Ethics oversight

Animal experiments followed European directive 2010I63IEU for animal research and were approved by the Institutional Animal Care and Use Committees (IACUC) of Technische Universität München and the ethical review board of the government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany) and UK Home office. Experiments were conformed to Institutional guidelines in Klinikum der Universität München/Ludwig Maximilian University of Munich).

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Recruitment

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Study protocol

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Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

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x	☐	Crops and/or livestock
x	☐	Ecosystems
x	☐	Any other significant area

Experiments of concern

Does the work involve any of these experiments of concern:

No	Yes	
x	☐	Demonstrate how to render a vaccine ineffective
x	☐	Confer resistance to therapeutically useful antibiotics or antiviral agents
x	☐	Enhance the virulence of a pathogen or render a nonpathogen virulent
x	☐	Increase transmissibility of a pathogen
x	☐	Alter the host range of a pathogen
x	☐	Enable evasion of diagnostic/detection modalities
x	☐	Enable the weaponization of a biological agent or toxin
x	☐	Any other potentially harmful combination of experiments and agents

ChIP-seq

Data deposition

☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links
May remain private before publication.

Files in database submission

Genome browser session
(e.g. UCSC)

Methodology

Replicates

Sequencing depth

Antibodies

Peak calling parameters

Data quality

Software

Flow Cytometry

Plots

Confirm that:

☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Instrument

Software

Cell population abundance

Gating strategy

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type

Design specifications

Behavioral performance measures

Acquisition

Imaging type(s)

Field strength

Sequence & imaging parameters

Area of acquisition

Diffusion MRI ☐ Used ☐ Not used

Preprocessing

Preprocessing software

Normalization

Normalization template

Noise and artifact removal

Volume censoring

Statistical modeling & inference

Model type and settings

Effect(s) tested

Specify type of analysis: ☐ Whole brain ☐ ROI-based ☐ Both

Statistic type for inference
(See Eklund et al. 2016)

Correction

Models & analysis

n/a	Involved in the study
☐	☐ Functional and/or effective connectivity
☐	☐ Graph analysis
☐	☐ Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Graph analysis

Multivariate modeling and predictive analysis