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Editorial Summary: This protocol describes how to perform CUBIC (Clear, Unobstructed Brain/Body Imaging Cocktails and Computational analysis), a simple and efficient method for organ clearing, imaging by light-sheet microscopy, and quantitative imaging analysis.

Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging

Keywords

CUBIC, Whole-brain and whole-body clearing, Immersion protocol, CB-Perfusion protocol, Light-sheet fluorescence microscopy (LSFM), Image informatics

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Abstract/summary

Here we describe a protocol for advanced CUBIC (*C*lear, *U*nobstructed *B*rain/*B*ody *I*maging *C*ocktails and *C*omputational analysis). The CUBIC protocol enables simple and efficient organ clearing, rapid imaging by light-sheet microscopy, and quantitative imaging analysis of multiple samples. The organ/body is cleared by immersion for 1-14 d, with the exact time required dependent on sample type and experimental purposes. A single set of imaging can be completed in 30-60 min. Image processing and analysis can take less than 1 d but is dependent on the number of samples in the dataset. The CUBIC clearing protocol can process multiple samples simultaneously. We previously used CUBIC to image whole-brain neural activities at single-cell resolution using Arc-dVenus transgenic (Tg) mice. CUBIC informatics calculated the Venus signal subtraction, comparing different brains at a whole-organ scale. These protocols provide a platform for organism-level systems biology by comprehensively detecting cells in a whole organ or body.

(149 words)

INTRODUCTION

Since the discovery of the 'cell' as the basic unit of living organisms, people have been seeking a way to observe all cells inside the body. Comprehensive analysis of cells in organs and whole organisms is expected to provide information about type, position, number and activity of cells and cellular networks. Tissue clearing followed by three-dimensional (3D) imaging is one approach that enables the analysis of multiple cells simultaneously in organs. Thus development of this and related technologies has become a recent trend[1, 2].

Development of tissue clearing methods

Early tissue clearing methods used organic chemicals (e.g. Benzyl alcohol-methyl salicylate, BABB, 3DISCO) for this purpose[3-6]. Some of these methods achieved high transparency within a few days by removing lipids and homogenizing refractive indices (RI) of the tissue and were shown to be compatible with whole-mount immunohistochemical analysis[7]. However, concerns about the quenching of fluorescent proteins and safety issues led to further method development. Very recent publication addressed this issue, reporting that pH control and temperature during clearing are the critical points to stabilize fluorescent proteins[8]. Alternative techniques, such as Sca*l*e[9],

use a hydrophilic chemical urea, and more recently developed tissue clearing methods use other hydrophilic reagents, including SeeDB[10], *Clear*^{T}[11], or 2,2'-thiodiethanol[12, 13] and FRUIT[14]. These methods are easy, safe, and fluorescent signals are retained however have relatively low clearing capability. The introduction of CLARITY enabled both the fluorescence retention and high transparency by embedding a tissue into hydrogel polymer and removing most of the lipids by electrophoresis[15]. Possible drawbacks of CLARITY included its technical difficulty and the limited scalability due to the need to use a specific device. However, these difficulties have been addressed by the development of passive clearing protocols that increased the scalability[16, 17].

In this protocol we describe how to perform CUBIC. CUBIC offers a high-performance and device-free tissue clearing method that preserves fluorescence based on hydrophilic reagents. It enables reproducible whole-organ and whole-body clearing. We have used CUBIC for clearing and rapid 3D imaging of whole mouse brains, a whole marmoset hemisphere, whole mouse organs (e.g. lung and heart) and whole mouse body. These images were used for image analyses for extracting biological information[18, 19].

Methods to image cleared tissues

Tissues cleared using the above methods can be imaged in 3D with optical microscopies. Because some of the above clearing methods render tissues highly transparent, light-sheet fluorescence microscopy (LSFM) has also been used for imaging[1, 2]. This type of microscopy can collect Z-stack images in a rapid manner and has been applied to 3D and 4D imaging, such as a time-lapse imaging of developing embryos or whole-brain calcium dynamics[20-23]. One of the earliest cases of whole mouse brain imaging was rapid whole-brain imaging of a BABB-cleared brain using a macrozoom-compatible light-sheet unit (Ultramicroscopy)[4]. More recently, COLM (CLARITY-optimized light-sheet microscopy) has been used for whole brain-scale imaging of CLARITY-processed brains[16]. Thus LSFM after an efficient tissue clearing method facilitates a high-throughput collection of multiple 3D images.

Rapid 3D imaging with LSFM can be used following whole-organ and whole-body clearing by CUBIC. CUBIC also provides processing and analysis of 3D images for extracting biological information. Therefore, CUBIC presents a platform of whole-organ/body imaging and image informatics, which enables a wide range of users to perform experiments targeting cellular and organ layers with multiple samples.

Development of CUBIC for efficient and reproducible whole-organ/body clearing

In developing a clearing technique for whole-brain and whole-body imaging we considered two main criteria, one, efficiency and transparency, and preservation of fluorescence for a rapid whole-brain/body imaging with LSFM, and two, reproducibility for comparative analysis of multiple samples. Because a clearing method with hydrophilic reagents had the potential to fulfill these criteria, we started by modifying the Sca*l*e recipe[9]. For this purpose, we constructed a new chemical screening method in which reduction of turbidity of a fixed-brain suspension was measured before and after mixing with a candidate chemical solution[18]. This screening enables non-biased discovery of brain clearing chemicals. We screened 40 Sca*l*e-related chemicals and found that aminoalcohols, in addition to urea and Triton X-100 in Sca*l*e, clear tissue with minimal fluorescent quenching[18]. In the CUBIC clearing protocol, we prepared two reagents Sca*l*eCUBIC-1 (reagent-1) and Sca*l*eCUBIC-2 (reagent-2) which also minimize light scattering inside the tissue. The first reagent works as a potential lipid remover. Lipid is thought to be the main light-scattering material inside tissue and its removal is correlated with the degree of transparency. A fixed brain was treated with the first reagent for about one week, washed with buffer and then immersed into the second reagent which has an RI close to ~1.49, similar to that of the SeeDB reagent[10]. Moving from buffer to the second reagent matched RIs between sample and reagent, which

1 further reduced light scattering within the tissue. Thus, a whole mouse brain became
2 transparent within ~14 d[18]. However, some chemicals seem to have additional or
3 different roles during the procedure, and thus further studies are needed to elucidate
4 tissue-clearing mechanisms.

5 In addition to light scattering, light absorption is another challenge in tissue
6 clearing. We accidentally found that aminoalcohols can remove heme in blood and
7 tissues[19], thus CUBIC is able to decolorize tissue. Perfusion of the CUBIC reagent
8 (CB-Perfusion) was used to efficiently penetrate the mouse body and to accelerate the
9 clearing and decoloring procedure. The CB-Perfusion protocol enabled not only faster
10 clearing of dissected tissues, but also whole-body clearing of infant and adult mice[19].

11 Because of its efficiency and reproducibility, the CUBIC protocol can be applied
12 to multiple samples in a single experiment, and is scalable from subcellular structures
13 (e.g. neuronal axons or spines) to marmoset brains to whole animal bodies[18, 19].
14 Furthermore, whole-organ counterstaining with a nucleic dye enables precise
15 positioning of genetically labeled cells in the whole-organ structure, extraction of
16 specific anatomical structures, and alignment of different samples for comparing signal
17 intensities[18, 19].
18

CUBIC for whole-organ/body imaging and image informatics

CUBIC-cleared samples can be used in LSFM. We use an optimized Ultramicroscope (LaVision BioTec) for this purpose. In rapid whole-organ imaging, a single, cleared, whole mouse brain can be imaged within 30-60 min per color and orientation. Fluorescence wavelength affects the quality of imaging results; in general, red wavelengths can penetrate deeper in tissue and thus better imaging results are obtained with red fluorescence than green fluorescence, particularly in deeper regions. To ensure weaker signals are detected in deeper regions, the sample is imaged in two orientations; in the case of whole-brain imaging, we took Z-stack images of dorsal side up (D-V) and ventral side up (V-D) directions.

Image visualization software such as Imaris can be used for depicting the reconstituted 3D image. Imaris implements numerous image analysis functions including spot counting and surface extraction. We performed extractions of anatomical structures in the 3D images for comparison of Langerhans islets in normal and diabetic pancreases, for example[19]. For more complicated analyses, we implemented image informatics often used in functional magnetic resonance image (fMRI) analysis[18]. First, structural images via counterstaining were registered to a reference brain to calculate transformation parameters. Then, the transformation parameters were applied to the

corresponding signal images (transgenes etc.) for alignment. These aligned images could be merged with each other to calculate signal subtraction between samples. This analysis was performed with open-source software such as Advanced Normalization Tools (ANTS)[24] and ITK-SNAP[25] but requires advanced informatics and computer science skills. For the user's convenience, we provide an easier analysis pipeline with prepared scripts in this manuscript (see below). As an example of the comparative analysis, we demonstrate 3D image analysis of Arc-dVenus Tg mouse brains with or without light stimulation and calculate the signal subtraction[18, 26]. The final subtraction data clearly depicts regions and cells in the whole brain where neurons responded to the light stimuli. Such direct comparative analysis by using whole-brain fluorescent 3D images was first reported using CUBIC informatics[18]. CUBIC informatics enables quantitative identification of stimulus- or timing-dependent neural activities and will help delineate structural abnormalities in disease samples at the whole-organ and whole-body scale.

Overview of CUBIC pipeline

CUBIC provides a platform for a comprehensive analysis of cells in a whole organ or body. Here we focus on describing: 1) the advanced CUBIC clearing protocols by

simple immersion and CB-Perfusion (steps 1-2); 2) whole-brain and -organ imaging with a LSFM (steps 3-6); and 3) CUBIC informatics for preprocessing and comparison of different brain samples (steps 7-15, see also **Fig. 1**). Although CUBIC is also applicable to staining with small chemicals or antibodies over days to weeks, as described previously[18, 19], we focus here on imaging of fluorescent proteins together with nuclear counterstaining.

Tissue clearing. Here we provide three clearing procedures: step 2 option A) simple immersion protocol for dissected whole organs; step 2 option B) CB-Perfusion and immersion protocol for faster clearing of whole organs; and step 2 option C) CB-Perfusion protocol for whole-body clearing. The immersion protocol in our first CUBIC report[18] has been improved to an advanced version (**Fig. 2**), in which the clearing speed and efficiency are increased. The CB-Perfusion protocol (**Fig. 3**) is almost identical to our second CUBIC report[19] but more detail is given in this manuscript. The clearing performance of CB-perfusion surpasses the immersion protocol, particularly in heme-rich organs (heart, muscle, kidney, or liver)[19] but tends to cause decreased signal intensity due to a short fixation time. Incubation period can be varied and is dependent on the organ and imaging methods to be used (**Fig. 1-3**). Users may select either of these options and determine the desired final transparency for their

experimental purposes. In either option, a paraformaldehyde (PFA) fixation is needed. Thus animals are transcardially perfused with 4% (wt/vol) PFA and then organs are dissected for post-fixation (step 1). Alternatively the fixed body can be further perfused with diluted reagent-1. Samples are subsequently treated with reagent-1 and reagent-2. Counterstaining is performed during and after reagent-1 treatment[18, 19]. Samples can be stored at various points in the procedure, indicated in the procedure as PAUSE POINTS.

***Whole-organ or whole-body imaging*.** We use a commercially available LSFM (Ultramicroscope, LaVision BioTec) supplied with an optimized macrozoom microscope (MVX-ZB10, Olympus) and an sCMOS camera (Andor NEO 5.5, 2560 × 2160 pixels). A customized sample holder is also used for larger brain and body samples and soft abdominal organs (**Fig. 4a**). A suitable pair of excitation laser and emission filters is also installed. We typically use 100 mW of 488 nm laser-ET525/50 emission filter for green, and 50 mW of 588 nm laser-ET650/60 emission filter for red to far-red fluorescence. The size of the acquired image per pixel is dependent on the zoom of the microscope so that one pixel is approximately ~5.2 × 5.2 μm at 2× zoom and one pixel is ~6.5 × 6.5 μm at 1.6× zoom. These pixel sizes are sufficient for detecting signal from single cells in regions such as the cerebral cortex, or even from subcellular structures when they were

sparsely labeled, as discussed below (**Fig. 4b-f, Supplementary Video 1**). Users can also use higher magnified zoom values (~6.3×) if finer resolution is needed. Z-step size is selected according to the thickness of laser sheet. We typically select the thinnest sheet of the used LSFM and set 10 μm as the Z-step size. For further image analysis as below, both signal images (e.g. transgenes) and structural images via whole-organ counterstaining should be collected. To ensure sharpness of signals throughout the 3D image, data of the same sample from different directions (D-V and V-D in the case of whole-brain imaging) are also collected. This is recommended because the ventral horizontal slices are sharper in V-D images and dorsal horizontal slices are sharper in D-V images (**Fig. 5**)[18].

If a proper LSFM is not available, widely used confocal or two-photon microscopies can be also used. Partially cleared samples by one-step immersion in reagent-1 for 1-3 d are even applicable to deep region imaging with a two-photon microscopy[18], because the clearing performance surpasses some of other clearing methods developed for these imaging purposes[19]. Microscopy venders have released objective lenses for deep tissue imaging (e.g. Olympus XLPLN10XSVMP, 10×/0.6, WD = 8 mm, ne = 1.33-1.52 and XLSLPLN25XGMP, 25×/1.0, WD = 8 mm, ne = 1.41-1.52;

Zeiss LD Plan-Aphochromat 20×1.0 WD = 5.6 mm, ne = 1.43-1.47; Leica HC FLUOTAR L25×/1.00, WD = 6 mm, ne = 1.457).

All raw image data collected in an uncompressed TIFF format (16-bit images for the LSFM, **Fig. 4b**) are typically about ~7 GB in total per brain/color/direction and thus 25-30 GB for a single brain dataset (structural/signal images and D-V/V-D directions). Therefore, a high-spec PC with a large memory size and good graphic board should be used for 3D reconstitution (**Fig. 4c-h**). We use a Windows PC with Intel(R) Core(TM) i7-3970X CPU @ 3.50GHz, 64 GB RAM and NVIDIA GeForce GTX 690 and Imaris software installed.

CUBIC informatics. Here we show the step-by-step procedures for data processing (steps 7-15). For preprocessing of raw data, each TIFF stack (**Fig. 5**, "Collect raw images") is first converted to a 3D image in the NIfTI-1 data format (.nii extension) introduced by the Neuroimaging Informatics Technology Initiative (NIfTI) (http://nifti.nimh.nih.gov/nifti-1/). NIfTI-1 files are visualized using software such as ITK-SNAP[25]. Due to the memory limitations of the current software tools, files need to be downscaled to 25% by discarding three of every four images of the TIFF *Z*-stack series and changing the resolution of these images from 2560 × 2160 to 640 × 540 (**Fig. 5**, "Downscaling"). This limitation should be overcome with future development of image

informatics tools. The downscaling procedure is done using ImageMagick (http://imagemagick.org/) to create a temporary stack of 16-bit PNG files for each original TIFF stack. Then, each PNG stack is converted to a NIfTI-1 file using the Convert3D tool from ITK-SNAP (**Fig. 5**, “Convert to NIfTI-1”). In this step, specification of the correct spacing (given the pixel number of raw image data and downscaling parameters) and the orientation (which depends on the acquisition direction) is needed.

In preparation for merging NIfTI-1 data in the same color channel of the same brain acquired from opposite directions (D-V and V-D), the files need to be aligned (**Fig. 5**, "Align D-V and V-D"). In this step, a pair of NIfTI-1 data images, the structural and signal images, is processed. The structural image is used to calculate the transformation parameter which is needed to align the D-V image to the V-D image. This is calculated by the *ANTS* function of the ANTS software. The transformation can allow deformation (e.g. Symmetric Normalization) or be restricted to affine operations only. Then, using the *WarpImageMultiTransform* function in ANTS, we apply the transformation parameters to align both structural and signal images of the D-V image to the V-D image.

Next, we merge the aligned images in order to ensure sharpness throughout the resulting 3D image (**Fig. 5**, "Combine D-V and V-D"). To do so, we use the Prewitt

operator[27] to calculate the "edge content" of the two images (as a proxy for image sharpness). This is used to define two thresholds, *n* and *m,* so that slices below *n* only come from the V-D image, slices above *m* only come from the D-V image, and slices in between the two are a linear combination of the two images. We then take the image pairs and the thresholds, and create the merged NIfTI-1 image. In order to access the values of individual pixels in the images, we use the *fsl2ascii* and *fslascii2img* functions of FSL[28]. The steps above produce a pair of D-V + V-D composite NIfTI-1 data for both structural and signal images.

Then, to facilitate analysis across different brain datasets, we align these merged NIfTI-1 files, which permits subtraction of signal images to be calculated. We show an example of Arc-dVenus Tg mouse brains with or without light stimuli[18], which express a destabilized version of yellow fluorescence protein Venus under control of the *Arc* gene promoter[26] (**Fig. 6a**). First, the raw data are preprocessed as in **Fig. 5**. Then, the composite NIfTI-1 images from different samples are aligned (**Fig. 6b**). As before, images are processed in pairs (structural and signal images), and the process relies on *ANTS* and *WarpImageMultiTransform*. We first align all brain datasets from the same experiment to an internal reference (i.e. one of the brain images among the samples of that experiment). Then, the internal reference is registered to a brain atlas such as the

Allen Brain Atlas[29]. These calculations thus provide the aligned 3D images to an atlas (**Fig. 6c**). Finally, the signal channel images of different brains are compared. To do so, we normalize these aligned images so that the median intensity inside the brain is the same across all brains. Then, the *fslmaths* function is used to compare pairs of brains by subtracting one image from the other (**Fig. 6d**). We provide the set of scripts for all steps and a brief user guide as **Supplementary Data**, with up-to-date versions also available on a GitHub repository (https://github.com/SystemsResearch/CUBIC_nprot). On our website (http://cubic.riken.jp), we also share example raw tiff data of Arc-dVenus Tg mice shown in **Fig. 6** and NIfTI-1-converted Allen Brain Atlas data.

***Limitations of the current version of CUBIC*.** CUBIC was developed and optimized for whole-organ and whole-body imaging and informatics analysis developed to enable a comprehensive pipeline. Thus, there are several important advantages to CUBIC compared to other clearing methods. The first is the active tissue decoloring ability. This is mild to proteins, as opposed to the simple flushing or harsh decolorization methods with peroxidase or acetone used previously[30, 31]. This enables a wide range of applications for not only the brain but also other organs inside (**Table 1**).

Because aqueous reagents are used to clear tissue, and fluorescent signals are preserved, tissues can be imaged by fluorescence microscopies. To achieve

efficient clearing for LSFM application, the optimal CUBIC reagent comprises five chemicals (**Table 2**) and takes days to two weeks to process. However, the procedure can be modified for the user's purpose: for example if users plan to image by two-photon microscopy, a one-step immersion in reagent-1 for 1-3 d is sufficient[18].

The actual scalability of CUBIC has not yet been fully investigated. So far, we tested a hemisphere of infant marmoset, and infant and adult whole mice[18, 19] (**Table 1**). Clearing of these samples was efficient: for example, in the case of cleared infant mouse, internal structures of the brain could be imaged directly even through the skull[19]. However, we have not yet tested adult primate brains (e.g. marmoset), although we plan to investigate this in future studies. While clearing of larger adult primate brains might be more difficult, longer incubation times and CB-Perfusion may address this issue. The PACT/PARS protocol of CLARITY has apparently achieved a similar scalability and may be considered as an alternative.

Whether a tissue clearing method can be combined with particular dyes or stains is an important consideration when selecting the clearing method. Whole-organ nuclei staining for anatomical annotation, registration and image analyses has been achieved using the CUBIC clearing procedure[18, 19]. Although we did not test other variations of dyes, possible limitations on some labeling methods, particularly lipophilic

dye labeling (e.g. DiI and related dyes), may exist given that the CUBIC clearing reagents massively remove lipids. Dyes or proteins should be fixed by PFA before clearing. In this sense, fluorescent proteins fused to a membrane protein can be observed in the cleared tissue while lipophilic dyes such as DiI may not be readily fixed by PFA due to their chemical structures and thus may be removed during clearing. This may be a drawback to CUBIC, in which case other clearing methods (e.g. *Clear*^{T}[11], SeeDB[10], FRUIT[14]) should be considered.

Structural distortion has been carefully addressed in some of clearing methods, such as SeeDB, and may need to be considered when clearing tissues with the other methods. Although we did not observe obvious changes in brain tissue even in the detailed subcellular structures including the axon and spine[18], such structural distortion may happen given that CUBIC reagents remove a large proportion of the lipids and cause transient swelling during the procedure. In addition, CUBIC has not yet been optimized to fully clear bone and melanin pigments (**Table 1**). However, this issue remains unaddressed by other clearing methods also, so we are unable to suggest a suitable alternative in this scenario. Thus this issue needs further investigation in future studies.

As discussed earlier, imaging resolution is a point to be considered as well. We have detected and counted signals from a single cell, and in this manuscript we therefore define this as providing 'single-cell resolution'. This criterion is roughly evaluated by using spot analysis of Imaris software in the dataset in **Fig. 4** (hippocampal cells of Thy1-YFP-H Tg) (**Supplementary Video 1**). In our opinion the criterion is overall supported by the calculated optical resolution. According to the vender's specifications, the optical resolution of microscopy that we use in this manuscript has 4.2 μm and 3.7 μm in X-Y images with 1.6× and 2× zoom, respectively. The thickness of light sheet is under 10 μm at the thinnest region, smaller than the typical step size (10 μm). In a typical nuclei-stained image, a 2.5 μm in half diameter sphere of a stained nuclei is detected as 2.5 + 3.7 μm in half diameter (actual half diameter + optical blurring of lens according to the definition of Rayleigh criterion) when 2× zoom (5.2 μm × 5.2 μm per pixel) is used. To distinguish two neighboring cells, two parameters need to be considered: Exclusion volume (EV), the average voxel volume per cell calculated from cell density; and s, the voxel volume of nuclei acquired by camera (considered both cell/nuclei diameter and optical blurring). Each nucleus is detectable and separable if EV is sufficiently larger than s. By our calculations, s = 3 × 3 × 2 voxels (= 15.6 × 15.6 × 20 μm = 4867.2 μm^3, enough to include a sphere with 2.5 +

3.7 μm in its half diameter). EV of mouse cerebral cortex neuron is 11000 μm^3/cell[32] = (22.2 μm)^3 = 5 × 5 × 3 voxels, which is sufficiently larger than the voxel volume used in this study, whereas EV of mouse hippocampal CA1 is 3900 μm^3/cell[33] = (15.7 μm)^3 = 3 × 3 × 2 to 4 × 4 × 2 voxels which is at the limit of resolution in the examples of current manuscript. Thus, the acquired image at its best has 'single-cell resolution' in the cortex and regions with similar cell density, while the voxel size is not sufficient for ‘single-cell resolution’ in denser regions such as the hippocampus or cerebellar granule layer. Further considerations are that the thinnest area of the sheet is limited and does not cover the entire imaged field. Also, the analysis software used in this manuscript does not support large image data and collected data must be downscaled. While CUBIC has the potential to detect all signals with single-cell resolution, these issues will need to be further addressed in future studies.

MATERIALS

REAGENTS

Animal samples used for imaging

- Animals expressing fluorescent proteins can be used. Strong expression of a bright fluorescent protein gives the best imaging results. A bright red fluorescent protein such as mKate2 is best. So far, we have confirmed good imaging performance with Thy1-YFP-H Tg (The Jackson Laboratory), R26-H2B-EGFP KI (RIKEN CDB), R26-H2B-mCherry KI (RIKEN CDB), R26-CAG-nuc-3×mKate2 KI (RIKEN CDB & QBiC), β-actin-CAG-nuc-3×mKate2 KI (RIKEN CDB & QBiC), CAG-EGFP Tg (Japan SLC,Inc.), and Arc-dVenus Tg (Gifu University). We also usually use C57BL/6 (CLEA Japan, Inc.) to prepare cleared organs and bodies. **! CAUTION** Animals experiments must be performed in accordance with governmental and institutional regulations regarding the use of animals for research purposes. All animal experiments and housing conditions in this manuscript were approved by the Animal Care and Use Committee of the RIKEN Kobe Institute, The University of Tokyo and the Gifu University, and all of the animals were cared for and treated humanely in accordance with the Institutional Guidelines for Experiments using animals.

Fixative, perfusion and storage reagents

- PBS tablets (Takara, cat. no. T9181)
- Heparin sodium (Mochida Pharmaceutical, 10000 U/10 mL)
- PFA (Nacalai Tesque, cat. no. 02890-45) **! CAUTION** PFA is toxic. Perform all procedures in a fume hood.
- HCl (Nacalai Tesque, cat. no. 18320-15 or 18321-05) **! CAUTION** HCl is toxic. Perform all procedures in a fume hood.
- NaOH (Nacalai Tesque, cat. no. 31511-05) **! CAUTION** NaOH is toxic. Perform all procedures in a fume hood.
- Sucrose (Nacalai Tesque, cat. no. 30403-55 or 30404-45)
- TISSUE TEK O.C.T. compound (Sakura Finetek, cat. no. 4583)
- 1/2 diluted Sca*l*eCUBIC-1 (1/2 reagent-1). Sca*l*eCUBIC-1 is mixed with an equivalent volume of distilled water. **▲CRITICAL** Sca*l*eCUBIC-1 should not be diluted with PBS, because contamination of salt decreases the clearing performance.

Clearing, nuclei-staining and imaging reagents

- *N,N,N',N'*-Tetrakis(2-hydroxypropyl)ethylenediamine (Quadrol, Tokyo Chemical Industry, cat. no. T0781)
- 2,2',2"-Nitrilotriethanol (triethanolamine, Wako, cat. no. 145-05605)
- Urea (Nacalai Tesque, cat. no. 35904-45 or 35907-15)

- Polyethylene glycol mono-*p*-isooctylphenyl ether (Triton X-100). **▲CRITICAL** In our first CUBIC paper[18], we mentioned that the quality of Triton X-100 product seems critical for preserving fluorescent signals and we recommended a product from Nacalai Tesque (cat. no. 25987-85). We further checked the same chemical from Sigma-Aldrich (cat. no. X100, T9284, T8787, T8532) or Tokyo Chemical Industry (cat. no. P0873), and neither of them caused fluorescent quenching in the final reagent-1 recipe in a short term and thus can be used as substitutes.
- Sucrose (Nacalai Tesque, cat. no. 30403-55 or 30404-45)
- Sodium azide (Nacalai Tesque, cat. no. 31208-82) **! CAUTION** Sodium azide is highly toxic.
- SYTO 16 (Life Technologies, cat. no. S7578)
- Propidium iodide (PI, Life Techologies, cat. no. P21493)
- Silicon oil TSF4300 (Momentive, RI = 1.498)
- Mineral oil (Sigma-Aldrich, RI = 1.467, cat. no. M8410)

EQUIPMENT

- 5-ml tube (Eppendorf, cat. no. 0030119.401SG)
- 15-ml conical tube (Corning, cat. no. 352096 or 188271)
- 30-ml conical tube (Sarstedt, cat. no. 60.544)

- 50-ml conical tube (Corning, cat. no. 352070 or 227261)
- Peristaltic pump (EYELA, model: MP-2000) (**Fig. 3a**)
- 23G intravenous injection needle, butterfly type (Terumo, cat. no. SV-23CLK)
- 26G×1/2” injection needle (Terumo, cat. no. NN-2613S)
- T shape stopcock (Terumo, cat. no. TS-TL2K)
- 1 ml, 10 ml and 20 ml disposable syringe (Terumo, cat. no. SS-01T, SS-1010SZ, SS-20ESZ)
- Vacuum desiccator (AS ONE, cat. no. VXS 1-5943-01) with vacuum pump (ULVAC, model: DA-15D) (**Fig. 2b**)
- Incubation devices. We use hybridization incubator (TAITEC, model: HB-80, **Fig. 2c**) or incubator (EYELA, model: FMS-1000 or MHS-2000) with rotator (TAITEC, model: RT-5)
- Shaker (TAITEC, model: Wave-PR or MixerXR-36, **Fig. 2d**)
- Magnetic stirrer for preparing highly viscous reagent (ASH, model: AMG-S, or IKEDA Scientific, model: IS-20PC)
- Hot stirrer for preparing highly viscous reagent (IKA, model: C-MAG HS10, or Advantec, model: SRS710HA)
- pH meter (HORIBA scientific, model: LAQUA twin)

- Positive displacement pipettor (Gilson, model: Microman M-1000). ▲**CRITICAL** We highly recommend this pipettor to measure the weight of the viscous materials such as Triton X-100 and aminoalcohols.
- Microwave
- Fume hood

Imaging microscopy for whole mouse organs

- Light-sheet illumination device with a macrozoom microscope[4, 18]

  In this study, we used Ultramicroscope from LaVision BioTec and MVX-ZB10 from Olympus, equipped with

- Olympus MVPLAPO0.63X lens (NA = 0.15, working distance = 87 mm)
- Imaging reservoir (100% quartz) (LaVision BioTec)
- Sample holder (LaVision BioTec or customized) (**Fig. 4a**)
- Green fluorescent signal filter (Chroma ET525/50)
- Red fluorescent signal filter (Chroma ET650/60)
- Coherent sapphire laser 488LP-100
- Coherent sapphire laser 588LP-50
- Andor sCMOS CCD camera Neo 5.5. The camera and the MVX microscope are connected to a camera adaptor (Olympus MVX-TV1X), tube lens (Olympus

MVX-TLU), and the Ultramicroscope filter wheel unit (LaVision BioTec) with adaptors (LaVision BioTec, LV AD MVX-1 and LV AD MVX-2)[18] (**Fig. 4a**).

- Customized sample holder
- Glass plate for specimen mounting stage

Image analyzing software

General image analysis tool

- ImageJ (freeware from the US National Institutes of Health (NIH))

Visualization tool

- Imaris (Bitplane, http://www.bitplane.com/imaris/imaris) for 3D reconstitution of TIFF image stacks
- ITK-SNAP (freeware from Paul Yushkevich, Ph.D. at the University of Pennsylvania and Guido Gerig, Ph.D. at the University of Utah, DL URL: http://www.itksnap.org/pmwiki/pmwiki.php) for NIfTI-1 3D images

Analysis tool

- Python and a C++ compiler
- Code provided as **Supplementary Data**
- ImageMagick (http://imagemagick.org/) installed with TIFF support

- Convert3D (http://www.itksnap.org/download/snap/process.php?link=7074&root=nitrc)
- ANTs 1.9-v4 (ANTs, freeware from stnava, DL URL: http://sourceforge.net/projects/advants/files/ANTS/ANTS_Latest/)
- FSL (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FslInstallation)

REAGENT SETUP

Phosphate buffered saline (PBS) Prepare according to vendor's manual. When using PBS tablets (Takara, cat. no. T9181), the tablet is dissolved in 1 liter of distilled water. When PBS/0.01% (wt/vol) sodium azide is prepared, directly dissolve 0.1 g of sodium azide in the 1 liter of PBS. This solution can be stored at room temperature (18–25 °C) for several months.

Paraformaldehyde (PFA) solution To prepare 4% (wt/vol) PFA in PBS, dissolve 40 g of PFA in 1 liter (total) of PBS. Heat the PBS solution (avoid boiling) and add PFA powder and 1/500-1/1000 volume of 1 N NaOH to help dissolving PFA faster. After complete dissolution, adjust the pH to 7.4 using HCl. PFA can be stored at -20°C until use for several months. ▲**CRITICAL** The pH value of PFA is a critical factor for an efficient clearing with lower autofluorescence. **! CAUTION** PFA is a very toxic reagent.

Avoid inhalation or contact with skin and eyes. Use a draft chamber, proper gloves and a mask to handle PFA, HCl, and NaOH.

80 wt% Quadrol Quadrol is a highly viscous liquid and can be used as an 80 wt% working solution. In this case, add 125 g of distilled water to a 500 g of Quadrol reagent bottle and stir for at least 30 min. Store the solution at room temperature for up to one month. ▲**CRITICAL** Quadrol is highly viscous and we use wt% rather than % (wt/vol) or % (vol/vol) for convenience.

Sca*l*eCUBIC-1 (Reagent-1) Reagent-1 is a mixture of urea (25 wt% final concentration), Quadrol (25 wt% final concentration), Triton X-100 (15 wt% final concentration) and distilled water. For example to prepare 500 g of reagent-1 solution, mix 125 g of urea, 156 g of 80 wt% Quadrol in 144 g of distilled water until complete dissolution at room temperature (or with heating if needed) and further add 75 g of Triton X-100. Finally, degas the reagent with a vacuum desiccator (~0.1 MPa, ~30 min) (**Fig. 2b**). The reagent can be stored at room temperature for up to one month. Prepare 1/2 reagent-1 by mixing 1:1 of reagent-1 and distilled water. ▲**CRITICAL** Quadrol and Triton X-100 are viscous and therefore we use wt% rather than % (wt/vol) or % (vol/vol) for convenience. Reagent-1 should not be prepared with PBS, because contamination of salt decreases the clearing performance. We usually use the stocked solution but longer

storage may cause quenching of fluorescent signals. Avoid excess heating during preparation

? TROUBLESHOOTING

Sca*l*eCUBIC-2 (Reagent-2) Reagent-2 is a mixture of urea (25 wt% final concentration), sucrose (50 wt% final concentration), triethanolamine (10 wt% final concentration) and distilled water. To prepare 50 g of reagent-2 solution, dissolve 12.5 g of urea and 25 g of sucrose in 7.5 g of distilled water with microwave and hot stirrer (**Fig. 2a**). After complete dissolution (typically it takes 10-15 min), cool the mixture at room temperature, add 5 g of triethanolamine, and stir further. The 0.1% (vol/vol) of Triton X-100 included in the original recipe[18] is not necessary. Finally, degas the reagent with a vacuum desiccator (~0.1 MPa, ~30 min) (**Fig. 2b**). Prepare 1/2 reagent-2 by mixing 1:1 of reagent-2 and PBS. Reagent-2 can be stored at room temperature for up to two weeks. **▲CRITICAL** Reagent-2 becomes highly viscous and therefore we use wt% rather than % (wt/vol) or % (vol/vol) for convenience. Because water evaporation will make it difficult for highly concentrated chemicals to dissolve, the weight should be monitored for the addition of evaporated water after completely dissolving urea and sucrose. Avoid boiling during the preparation. **▲CRITICAL** Reagent-2 should not be prepared with PBS, because contamination of salt decreases the clearing performance. We use PBS

only in preparing 1/2 reagent-2/PBS, because tissues after reagent-1 treatment tend to be easily swollen in 1/2 reagent-2/water, which might cause distortion of overall structure, and because the gradual exchange from PBS through 1/2 reagent-2/PBS to salt-free reagent-2 does not affect the final transparency. Before clearing, 1/2 reagent-1/water is not a problem. **▲CRITICAL** Make sure that there is no precipitation in the reagent-2 solution stock before use. The precipitation in the stock can be dissolved again by mild heating with microwave. Insufficient degassing may cause bubbles around and inside the tissue during reagent-2 treatment. **! CAUTION** The acrid ammonia smell in these reagents indicates degradation of urea. Generation of ammonia itself is not apparently a problem because the reagents are buffered with aminoalcohol in alkaline pH range (~pH 11)[19]. We recommend users to avoid excess heating during preparation. If users experience the smell during clearing, change to the fresh media.

? TROUBLESHOOTING

Immersion oil mix Mix 1:1 of TSF4300 and mineral oil completely with stirrer and degas with a vacuum desiccator (~0.1 MPa, ~30 min) (**Fig. 2b**) before use. The oil mix can be repeatedly used for imaging by filtering contaminants (clearing reagents etc.). Its RI is 1.48-1.49, a comparable RI of reagent-2. The oil can be wiped out with 70% EtOH.

▲**CRITICAL** The mix ratio can be optionally changed because the best RI matching may be different between organs.

EQUIPMENT SETUP

Surgical setup for CB-Perfusion protocol Typical surgical setup for CB-Perfusion is depicted in **Fig 3a**. In the protocol, the adult mouse is perfused with four solutions:(1) 20-30 ml of cold heparin-PBS, (2) 150 ml of cold 4% (wt/vol) PFA, (3) 20 ml of PBS, (4) 20-30 ml of 1/2 diluted reagent-1. We recommend that PFA is perfused by peristaltic pump for successful and reproducible surgeries. Thus, we devised a surgical instrument with a combination of T shape stopcocks, a peristaltic pump connected with silicon tube, an intravenous injection needle, and a disposable syringe as shown in **Fig 3a**. **!** **CAUTION** PFA is a very toxic reagent. Avoid inhalation or contact with skin and eyes. Use a draft chamber, proper gloves and a mask to handle PFA. Great care in handling the injection needle is needed to avoid accidental needlesticks.

PROCEDURE

Anesthesia ● TIMING 5 min

1| At day 0, deeply anesthetize the mice using pentobarbital (~150 mg/kg of body weight, administer intraperitoneally with 1 ml syringe and 26G×1/2” injection needle).

! CAUTION Every experiment must follow all government and institutional guidelines for the use of experimental animals.

Transcardial perfusion and tissue clearing ● TIMING 4-14 d

▲**CRITICAL** In this step, we particularly focus on the full clearing protocol for the purpose of LSFM imaging. However, the immersion period and the final transparency of samples can be varied according to user’s experimental purpose.

2| Start clearing organs by simple immersion protocol (option A) or CB-Perfusion and immersion protocol (option B) (CB-Perfusion clears better than the immersion protocol, particularly in heme-rich organs, but tends to cause decreased signal intensity due to the shorter fixation time). Alternatively start clearing a whole-body by the CB-Perfusion protocol (option C).

▲**CRITICAL STEP** Option A is for a single whole mouse brain (**Fig. 2**) and may need some modifications when other organs are cleared. Because handling of whole-body

samples in the viscous reagent-2 become difficult, particularly due to causing bubbles, the cleared whole body with option C is kept in reagent-1 but not in reagent-2.

(A) Simple immersion protocol for dissected whole brain

(i) Day 0: Perfuse the mice with 10 ml of cold PBS (pH 7.4) containing 10 U/ml of heparin at ~10 ml/min to remove the blood from the organ as much as possible. Then, perfuse ~25 ml of cold 4% (wt/vol) PFA (pH 7.4) at ~10 ml/min. Dissect the brain and postfix in 10 ml of 4% (wt/vol) PFA with shaking at 4°C for 18-24 h.

▲**CRITICAL STEP** Cooling of PBS and 4% (wt/vol) PFA on ice is important for successful perfusion. Muscle stiffness after perfusion is a good indicator of successful perfusion. Residual blood in the mouse brain increases autofluorescence especially in the green-laser excitation. The pH value of PFA is also critical for efficient clearing and lower autofluorescence. Overfixation causes both lower clearing efficiency and autofluorescence.

? TROUBLESHOOTING

(ii) Day 1: wash the tissue sample with 10 ml of PBS/0.01% (wt/vol) sodium azide for at least 2 h twice at room temperature to remove the remaining PFA. (**Fig. 2e**, “Fixed brain” panel)

■ **PAUSE POINT** The fixed organs can be stored. First immerse them in 10 ml of 20-30% (wt/vol) sucrose in PBS per organ with shaking at 4°C for 1-2 d. When samples sink to the bottom, put them into O.C.T. compound and immediately transfer to –80°C. To continue the clearing protocol, thaw samples gradually at room temperature, wash with PBS at least twice, with each wash for 1 h, to remove sucrose and O.C.T. compound. The sample will now be ready for the next step, however, we find the clearing efficiency is reduced in samples that have been stored.

(iii) Immerse the sample in 8-10 ml of 1/2 water-diluted reagent-1 with shaking (~60 r.p.m. if using the orbital shaker of a hybridization oven in **Fig. 2c**, ~30 r.p.m. if using a seesaw shaker in **Fig. 2d**) or rotation (~5 r.p.m.) at 37°C for 3-6 h. We recommend using a 30-ml of conical tube for clearing a single brain rather than a 15-ml tube in this and further clearing steps (iv-v) because of sample swelling. Clearing effects can be observed during this step (**Fig. 2e**, “1/2 reagent-1” panel). Note that a nuclear staining dye, such as SYTO 16 (1-2 μM) and PI (5-10 μg/ml), can also be added to 1/2 diluted reagent-1 at this step.

▲**CRITICAL STEP** Inefficient mixing of the reagent and samples during clearing may affect the final clearing performance. Pretreatment with 1/2 diluted reagent-1

gives a more effective final clearing efficiency than direct immersion in reagent-1. However, this step can be skipped for other purposes such as two-photon imaging with a partially cleared sample, for example[18]. This direct immersion procedure gives better clearing results than some other clearing methods[19] and can be used for two-photon imaging[18] and possibly as part of the sample preparation for single-photon imaging.

(iv) Discard 1/2 diluted reagent-1. Immediately add 8-10 ml of reagent-1 and gently shake or rotate the sample at 37°C overnight. If desired, the same concentration of nuclear staining dye used in the previous step should be added to reagent-1.

! CAUTION Reagent-1 can erase oily pen marks easily. Make sure the tube is sealed by wrapping parafilm around the lid/top of it. Sample labels should be written on both the body and lid of the tube to avoid loss of information. An ammonia smell indicates degradation of urea, and that the reagent should be replaced by fresh media.

? TROUBLESHOOTING

(v) Day 2: replace 8-10 ml of reagent-1 and continue gentle shaking or rotating at 37°C. Replace the reagent every two days (day 4 and 6). Also refresh any nuclear

staining dye on day 4 and day 6. Typically, the brain will be sufficiently cleared by day 7-8 (**Fig. 2e**, “Reagent-1” panel).

▲CRITICAL STEP If the white matter has not significantly cleared by 8-day immersion, try further immersion by placing into fresh reagent-1 for an additional 1-2 d.

! CAUTION Since CUBIC-treated organs soften, we recommend using spoons instead of forceps for handling them in order to avoid damage.

? TROUBLESHOOTING

(vi) Day 7-10: To stop the clearing procedure, wash the sample with 20 ml of PBS/0.01% (wt/vol) sodium azide with gentle shaking or rotating at room temperature for at least 2 h × 3 times. We typically wash the sample for 2 h × 1, overnight × 1 and 2 h × 1. When a sample is stained with PI, further staining during this step is needed: thus incubate the washed sample in ~5 ml of PBS/0.01% (wt/vol) sodium azide containing 5-10 μg/ml of PI for additional 3 d (or more, if needed) at 37°C with rotation[18].

▲CRITICAL STEP Complete removal of reagent-1 during the washing step is critical for final clearing efficiency.

■ **PAUSE POINT** Organs can be stored. First immerse them in 10 ml of 30% (wt/vol) sucrose in PBS/0.01% (wt/vol) sodium azide per organ with shaking at room temperature overnight. When samples sink to the bottom, put them into O.C.T. compound and immediately store them at –80°C. Thaw as described in the PAUSE POINT at step (ii).

! CRITICAL STEP For cryoprotection at this step, we recommend using 30% (wt/vol) sucrose in PBS rather than 20% (wt/vol) sucrose solution to avoid any damage to the sample.

? TROUBLESHOOTING

(vii) Day 8-11: Degas the sample in a limited volume of PBS with a vacuum desiccator (**Fig. 2b**). To do this, immerse the sample in ~5 ml of 1/2 PBS-diluted reagent-2 and shake it in 5 ml tube for 6 h to 24 h at 37°C or room temperature (**Fig. 2e**, “1/2 reagent-2” panel). Check whether the sample sinks to the bottom (a sign of complete immersion).

▲**CRITICAL STEP** Degassing of sample prevents air bubbles from remaining in the ventricle.

(viii) Day 8-11: Immerse the sample in ~5 ml of reagent-2 in a 5 ml tube and gently shake at 37°C overnight. The next day, replace the reagent with fresh reagent and further incubate for ~24 h (**Fig. 2e**, “Reagent-2” panel).

! CAUTION Do not rotate the tube to avoid making bubbles. Samples do not sink in the highly viscous reagent-2 and it is difficult to take images in the reagent. The reagent-2-treated samples should be immersed in the low-viscous immersion oil mix at imaging steps. When structural distortion is apparent after reagent-2 treatment at 37°C, try incubation at room temperature for a longer time. Adjustment of PBS content in 1/2 reagent-2 may also have an effect to mitigate unsuited shrinkage or swelling.

■ **PAUSE POINT** Organs can be left in reagent-2 for up to one week at room temperature. Further immersion increases the final transparency but also causes swelling of the sample. After imaging, the sample can be washed with PBS/0.01% (wt/vol) sodium azide, completely immersed in 30% (wt/vol) sucrose in PBS/0.01% (wt/vol) sodium azide, and stored in O.C.T. compound at –80°C as described in the PAUSEPOINT to step (vi).

? TROUBLESHOOTING

(B) CB-Perfusion and immersion protocol for faster clearing of whole organs

(i) Prepare the surgical setup as shown in **Fig. 3a**.

(ii) Day 0: Perfuse the mice with 20-30 ml of cold PBS (pH 7.4) containing 10 U/ml of heparin at ~10 ml/min to remove the blood from the tissues as much as possible.

▲CRITICAL STEP Insufficient removal of blood inside the tissue prolongs the clearing period and may cause low clearing performance.

(iii) Perfuse the mice with 150 ml of cold 4% (wt/vol) PFA in PBS (pH 7.4) at ~15 ml/min using a peristaltic pump.

! CAUTION PFA is a very toxic reagent. Perform all procedures in a fume hood with a safety glass to avoid inhalation or contact with skin and eyes.

▲CRITICAL STEP If the signal from a target reporter protein is weak, a prolonged perfusion period may be more effective, or clearing as described in option A.

▲CRITICAL STEP Cooling of PBS and 4% (wt/vol) PFA on ice is important for successful perfusion.

? TROUBLESHOOTING

(iv) Perfuse the mice with 20 ml of PBS (pH 7.4) at ~10 ml/min to wash out PFA, followed by perfusion of 20-30 ml of 1/2 diluted reagent-1 at the same injection rate. Make sure that organs become translucent by the end of the perfusion (**Fig. 3b**).

Note that a nuclear staining dye, such as SYTO 16 (1-2 μM) and PI (5-10 μg/ml), can also be added to 1/2 diluted reagent-1 at this CB-Perfusion step.

▲**CRITICAL STEP** Perfusion efficiency is crucial to the final clearing efficiency. Some organs such as the pancreas and spleen are good indicators to evaluate perfusion efficiency (**Fig. 3b**).

(v) Dissect the organs of interest and immerse these in reagent-1. Several organs can be processed in a single tube, but the stomach and intestine should be separated into different tubes. Gastrointestinal content in these organs should be removed as much as possible in this step. Thus immerse several organs such as heart, lung, kidney, spleen, pancreas and a piece of liver in 40 ml of reagent-1 or immerse each small organ such as heart, lung, kidney, spleen, and pancreas in 5 ml of reagent-1. Incubate the samples with shaking (~60 r.p.m. if using an orbital shaker in a hybridization oven in **Fig. 2c**, ~30 r.p.m. if using a seesaw shaker in **Fig. 2d**) or rotation (~5 r.p.m.) at 37°C overnight. Add the same concentration of any nuclear staining dye used at step (iv) to reagent-1.

▲**CRITICAL STEP** Efficient mixing of the reagent and samples during clearing may affect the final clearing performance. For efficient clearing, the samples of interest should be immersed in at least 5-fold volume of reagent-1.

! CAUTION Reagent-1 erases oily pen marks easily. Make sure the tube is sealed by wrapping parafilm around the lid/top of it. Sample labels should be written on both body and lid of the tube to avoid loss of information. An ammonia smell indicates degradation of urea, and in this scenario the reagent should be replaced with fresh media.

? TROUBLESHOOTING

(vi) Day 1: make sure that organs are transparent and the color of the supernatant has turned olive green. Replace reagent-1 with the same volume of reagent-1 and continue clearing shaking at 37°C. If appropriate, also refresh the nuclear staining dye. Replace reagent-1 and any nuclear staining dye again at day 2 and 4. The total incubation time for the complete clearing depends on the organ: 1-day of reagent-1 treatment is usually sufficient in the case of pancreas, spleen, and intestine. However, note that we treated all indicated organs in **Fig. 3c** with reagent-1 for 5 d.

▲CRITICAL STEP Typically, successfully CB-Perfused-organs are turned almost transparent with the exception of liver and lung by day 1 (**Fig. 3c**). Opacity in the lung occurs mainly from bubbles. Note that the color change of the supernatant indicates decolorization of tissues due to heme elution.

? TROUBLESHOOTING

(vii) Day 2-6: To stop the clearing procedure, wash the samples with same volume of PBS with gently shaking or rotating at room temperature for 2 h × 3 times. After the PBS wash, move to the next step immediately.

! CRITICAL STEP CB-Perfused samples are prone to overshrink in the washing step. Do not wash the samples in PBS more than 3 times × 2 h.

(viii) Immerse the sample in the same volume of 1/2 PBS-diluted glycerol and shake for 6 h to 24 h at room temperature. Check whether the sample sinks to the bottom (a sign of complete immersion).

(ix) Day 3-7: Immerse the samples in the same volume of reagent-2 and gently shake at 37°C overnight. The next day, replace the reagent with fresh reagent and further incubate for several days. Typically, an apparent transparency plateau is reached after 2-3 d of reagent-2 treatment. At day 10, almost all organs should be transparent as shown in **Fig. 3c**. The gastrointestinal content in stomach and intestine should be removed as much as possible before the following imaging step.

! CAUTION To avoid making bubbles, do not rotate the tube. Samples do not sink in the highly viscous reagent-2 and it is difficult to take images in the reagent. The

reagent-2-treated samples should be immersed in the low-viscous immersion oil mix at imaging steps. When structural distortion is apparent after reagent-2 treatment at 37°C, try the incubation at room temperature for a longer time. Or, test the simple immersion protocol (step **(A)**).

? TROUBLESHOOTING

■ **PAUSE POINT** Tissues can be left in reagent-2 for up to one week at room temperature. Further immersion increases the final transparency but also causes swelling of the sample. After imaging, the sample can be washed with PBS, completely immersed in 30% (wt/vol) sucrose in PBS, and stored in O.C.T. compound at –80°C, as in the PAUSE POINT for step **(A)** (vi)

(C) CB-Perfusion protocol for whole-body clearing

▲**CRITICAL** Here, we describe clearing of whole adult mouse body only using reagent-1. This overcomes the difficulties of handling whole-body samples in the viscous reagent-2, particularly due to bubble formation. For infant mouse whole-body imaging, perfusion and immersion of reagent-1 was sufficient (**Table 1**)[19]. Adult whole-body imaging is not applicable with the microscope setup introduced in this manuscript due to stage size limitations.

(i) (Perform CB-Perfusion as described in **option B)** steps (i)-(iv).

(ii) Detach the skin from the body. Carefully remove as much pelage as possible. Make sure that the body is partially transparent following the CB-Perfusion. Typically, glands such as pancreas and submaxillary gland are almost transparent (**Fig. 3d**). Spleen is also as a good indicator of successful perfusion. Immerse the body in 200 ml of reagent-1. Place the container on orbital shaker set at ~60 r.p.m. or seesaw shaker set at ~30 r.p.m. in the incubator at 37°C overnight. Use the same concentration of the nuclear staining dye used at step **B** (iv) and add to reagent-1 if desired.

▲**CRITICAL STEP** Inefficient mixing of the reagent and samples during clearing may affect the final clearing performance.

(iii) Replace the same volume of reagent-1 and continue gentle shaking at 37°C. Refresh the nuclear staining dye if used also. Replace the reagent (and any nuclear staining due) every day in the initial week, and every two or three days in the second week. Continue the clearing with reagent-1 for at least two weeks. Typically, major abdominal organs except bones and intestinal content become sufficiently transparent after two weeks of reagent-1 treatment (**Fig. 3d**).

▲**CRITICAL STEP** An ammonia smell indicates degradation of urea, and the reagent should be replaced with fresh media in this case.

■ **PAUSE POINT** The whole body can be kept in reagent-1 for up to several months at room temperature.

? TROUBLESHOOTING

Imaging of the cleared tissues with the macrozoom LSFM ● **TIMING 1-3 h/sample,** timing is dependent on the number of samples, color and direction as well as required exposure time.

▲**CRITICAL** To perform a rapid image acquisition of whole organs, a light-sheet illumination unit combined with a macrozoom microscope is suitable. Here we describe our setup using Ultramicroscope/MVX-ZB10 (LaVision BioTec/Olympus). A confocal or a multi-photon microscopy can be also applied but for more limited regions.

3| Before imaging, wipe reagent-2-treated samples with a kimwipe softly to remove excess reagent-2 on the surface and then immerse the sample into the oil mix for 10 min to 1 h. This process also helps remove bubbles around the tissue. If bubbles attach on the surface of the sample, carefully remove them with a needle or a tapered forceps.

4| Set the imaging reservoir filled with the immersion oil mix, and then set the sample holder. Put a glass slide on the sample holder (**Fig. 4a**).

5| Put the sample on the glass slide (**Fig. 4a**). Acquire a live image with an appropriate laser/filter pair to adjust focus and the sample position to the center. A whole mouse brain image can be captured using 1.6× to 2× zoom of the MVX-ZB10.

▲CRITICAL STEP To avoid fluorescent quenching, laser power should be weakened during the adjustment of position and focus.

6| Set the focus position of illumination sheet, Z-range (in the case of whole brain, typically ~7 mm in total), Z-step size (typically 10 μm per step), laser power (typically 70-100%) and exposure time (typically 50 ms to 1 s for each side). Each plane should be illuminated from both the right and left sides, and a merged image with max intensity saved. The exposure times should be adjusted according to the fluorescent signal intensities of each sample. After all parameters are adjusted, start image acquisition. When multi-color images are needed, repeat the image acquisition procedure with the same Z-range and re-adjusted laser power and exposure time. For collecting both D-V and V-D datasets, manually flip the sample upside down and acquire image again for this opposite orientation.

▲**CRITICAL STEP** To take images with a high signal-to-noise ratio, it is important to use bright fluorescent proteins and chemicals. Because ~700 images (~11 MB per image, total ~7 GB) are acquired per color/direction, each image should be saved to a secondary storage (e.g. a hard-disc drive) during acquisition. Each stack (one color, one direction) is saved to a different folder. For further signal comparison steps (step 7-15), signal and structural images of both D-V and V-D directions are needed. We recommend a simple naming rule for these folders with four fields separated by an underscore: information about the experiment (including imaging date), a unique ID for this brain, information about the imaging direction ("VD" or "DV") and information about the channel ("nuclear" for the nuclear counterstaining and "geneExp" for the signal channel). For instance, 20131118LAdV_001_nuclear_DV. The code provided for the informatics section assumes that the naming convention is respected. It is also important to note that white spaces and special characters must be avoided.

? TROUBLESHOOTING

Informatics for signal comparison ● TIMING 8-9 h (for a 2-brain dataset, reduced to 1-2h if using affine registration instead of Symmetric Normalization)

▲**CRITICAL** We provide our source code and an additional user manual as **Supplementary Data**, as well as NIfTI-1-converted Allen Brain Atlas data and Arc-dVenus Tg mouse brain images used in **Fig. 6** on our website (http://cubic.riken.jp). Note that timing is roughly proportional to the number of brains and vary on different computer specifications.

7| If this is the first time the pipeline is being run, install all required software and copy the provided code to the computer used for the analysis. Compile the C++ files for edge detection (g++ -O3 edge_detection_Prewitt.cpp -o edge_detection_Prewitt) and file merging (g++ -O3 file_merging.cpp -o file_merging).

8| Construct a 3D NIfTI-1 file for each TIFF stack with the *convertTiffFiles.py* script. This takes approximately 2 min per stack, and each brain sample corresponds to four stacks (two channels, two acquisition directions).

? TROUBLESHOOTING

9| Align images of the same brain acquired from opposite directions, with the *sameBrainAlignment.py* script. For each brain, this includes: registration of the

V-D-acquired nuclear counterstaining image to the D-V-acquired one, alignment of the V-D-acquired nuclear counterstaining image, alignment of the V-D-acquired signal channel image. The registration takes approximately 1 h 30 min using Symmetric Normalization or 3-5 min using affine transformations only, and both alignments take under a minute.

? TROUBLESHOOTING

10| Merge the V-D-acquired and D-V-acquired images. Use *edgeDetection.py* to calculate the *n* and *m* thresholds for each brain and channel (4 min per brain), and use these results in *fileMerging.py* to merge the files (7-8 min per brain).

? TROUBLESHOOTING

11| Choose one brain to be used as internal reference, and align all the other brains to this reference with *internalAlignment.py* (for each brain, approximately 1 h 30 min for the registration if using Symmetric Normalization or 3-5 min if using affine transformation only, and under a minute for the alignment of both channels).

? TROUBLESHOOTING

12| Using *atlasAlignment.py*, register the internal reference to the brain atlas (approximately 1 h 30 min) and align all brains to the atlas (approximately 1 min per brain).

? TROUBLESHOOTING

13| Calculate the normalization factors (7 min, plus 7 min per brain) with *median_brainOnly.py*.

? TROUBLESHOOTING

14| Normalize and compare brains (e.g. for subtraction) with *normalisation_comparison.py* (exact timing dependent on comparison, but typically 10-60 min).

? TROUBLESHOOTING

15| Export TIFF stack for the resulting files with *exportTiffStack.py* (about four min per NIfTI-1 file).

▲CRITICAL STEP Although registration with SyN transformation is effective, in some cases it may cause structural deformation or distortion. Users can choose whether to

use SyN or affine-only registration. We recommend checking the quality of final aligned data and, if necessary trying SyN or affine-only instead. In this manuscript, we show data aligned with SyN in **Fig.5** and with affine-only transformation in **Fig.6**, for illustrative purposes.

? TROUBLESHOOTING

TROUBLESHOOTING

See Table 3 for troubleshooting guidance

ANTICIPATED RESULTS

The CUBIC pipeline can be used for whole-organs or -body clearing. It is simple, efficient, and reproducible and thus can be applied to simultaneous multi-sample clearing in a single tube (**Fig. 2c**, inset) or a plastic container. The procedure can be performed using equipment usually used in a typical biology laboratory (**Fig. 2a-d**). By simple immersion of sample in reagent-1, clearing is obvious within several hours (**Fig. 2e**) and such partially cleared samples are even applicable to deep region imaging with two-photon microscopy[18]. The CUBIC reagents also decolorize organs and the whole body by removing heme, which is also apparent just after CB-Perfusion (**Fig. 3a** and **b**), and this ability enables whole-body clearing within two weeks (**Fig. 3c** and **d**). We summarize the organs we have used CUBIC on in **Table 1**. Removal of the CUBIC reagents by PBS wash reverses the cleared state (**Fig. 2e**), but the tissue is clear again if re-immersed into CUBIC reagents.

Fig. 4a-f shows typical imaging results for the Thy1-YFP-H Tg mouse brain at 1.6× zoom. Overall, soma and other subcellular structures such as neurites in the mouse brain can be captured providing they are sparsely labeled (**Fig. 4b, d, e, Supplementary Video 1**). Other organs were also subjected to rapid, multi-color 3D

imaging in the same platform (**Fig. 4g** and **h**). Of note, these data were collected for approximately 30-60 min for each direction/color.

According to the user's experimental purpose, high or low resolution images may be acquired. We use relatively low resolution because our primary purpose is to analyze cells within the context of a whole organ or body in a high-throughput manner. However subcellular structures can be observed in the cleared tissue (**Fig. 4b-f**)[18] and thus CUBIC permits more detailed observations with a higher zoom on the LSFM, or by using higher NA objective lenses on other confocal and two-photon microscopes .

We implemented an image informatics method originally used in fMRI analysis to compare different brains. In this pipeline, the acquired dataset are preprocessed by merging D-V and V-D to make sure the resulting images are clear throughout the Z-stack (**Fig. 5**). Then signal subtraction between samples is calculated (**Fig. 6**). Here we show an example of Arc-dVenus Tg mouse brains with or without light stimuli[18, 26] (**Fig. 6a**). The raw images were preprocessed, aligned and normalized, and then subtraction of Venus signal was calculated to visualize light-responsive regions at the whole-brain scale (**Fig. 6b-d**). These calculations could be achieved by whole-organ counterstaining in the CUBIC clearing protocol. Note that the final resolution of images in this process is downscaled and it is more difficult to achieve 'single-cell resolution'

throughout the entire imaging field, due to the current software limitations. This will be addressed in future studies.

In summary, CUBIC provides a platform for comprehensive cell detection and analysis across whole organs and the body. Possible applications of CUBIC will be for whole-organ samples from multiple conditions or timepoints, detection of aberrant 3D morphological changes in diseased tissues or a scalable observation of tissue-to-subcellular structures in a single cleared organ. The method will therefore support organism-level systems biology and facilitate our understanding of complicated biological phenomena in multicellular organisms.

FIGURE LEGENDS

Figure 1

Overview of the CUBIC pipeline. CUBIC is composed of 3 major stages (clearing, imaging, and analysis). For efficient and reproducible clearing, we provide three protocols: 1) simple immersion (step **2A**) which takes ~11 d in the case of a whole brain from an adult mouse (but varied according to the experimental purpose and organs), 2) CB-perfusion protocol for the whole adult mouse (step **2C**), which takes ~14 d, 3) the CB-Perfusion and immersion hybrid protocol (step **2B**) in which dissected organs after CB-Perfusion can be continuously cleared according to the simple immersion protocol. Rapid 3D imaging can be performed with an LSFM. The collected data is processed and analyzed, such as signal comparison between samples as described in this manuscript. Images of actual samples correspond to the samples in **Fig. 2** and **3**. All animal experiments here were approved by the Animal Care and Use Committee of the RIKEN Kobe Institute and The University of Tokyo, and all of the animals were cared in accordance with the Institutional Guidelines.

Figure 2

Procedure of the simple immersion protocol. **(a)** Preparation of reagent-2. This reagent contains a high concentration of urea (25 wt%) and sucrose (50 wt%) (upper panel). These can be completely dissolved by heat and stirring with a microwave and a hot stirrer (lower panel). **(b)** A vacuum desiccator for the degas steps. **(c)** An incubator with shaker (a hybridization incubator) that we use for the clearing procedure. Inset: 5 brain samples treated with reagent-1 in a single tube (day 5). **(d)** A table shaker used for PBS washing step at room temperature. **(e)** Appearance of a brain sample in each step. A brain from C57BL/6 male mouse (8-week-old) was used. Reagent-1-treated sample is temporally swollen but the size is recovered after immersion in reagent-2. Scale bar: 5 mm. All animal experiments here were approved by the Animal Care and Use Committee of the RIKEN Kobe Institute and The University of Tokyo, and all of the animals were cared in accordance with the Institutional Guidelines.

Figure 3

Procedure of the CB-Perfusion protocol. **(a)** Surgical setup for CB-Perfusion. The transcardiac perfusion line is connected to 1) heparin-PBS for flushing the blood, 2) 4% PFA on ice with peristaltic pump for fixation, 3) PBS for flushing PFA, and 4) 1/2-diluted reagent-1 for accelerative clearing through the vascular system. **(b)** Dissected organs

just after CB-Perfusion. Organs such as the pancreas, spleen or kidney are macroscopically cleared and decolored at this point. The clearance of these organs is indicative of how well researchers succeed in CB-Perfusion. Scale bar: 5 mm. **(c)** Clearing performance of CB-Perfused dissected organs at day 1 in reagent-1 and at day 10 in reagent-2. CB-Perfused organs with a successful procedure are significantly transparent after 1 d of reagent-1 treatment. Some of the organs such as pancreas are more transparent in reagent-1 rather than in reagent-2. Scale bar: 5 mm. **(d)** Clearing performance of a CB-Perfused whole body just after CB-Perfusion and after two weeks of reagent-1 treatment. Organs such as pancreas, submaxillary gland and spleen tend quickly become transparent by CB-Perfusion. Major abdominal organs except bones and gastrointestinal content become sufficiently transparent after two weeks of reagent-1 treatment. C57BL/6 male mice (8-week-old) were used in **(b)-(d)**. All animal experiments here were approved by the Animal Care and Use Committee of the RIKEN Kobe Institute and The University of Tokyo, and all of the animals were cared in accordance with the Institutional Guidelines.

Figure 4

Whole-organ imaging with LSFM. **(a)** The microscope setup and the customized sample holder (inset) used in this manuscript. **(b)** Left: a raw TIFF image (2560 × 2160) from a cleared Thy1-YFP-H Tg mouse brain[34] (male, 23-week-old) (imaging conditions: Z = 10 μm step × 749 planes, zoom = 1.6×, expose = 50 ms × two illuminations from each side, total acquisition time = about 30 min). The sample was cleared according to the immersion protocol in this manuscript. Right: a magnified image of the indicated area in the left panel. These images were minimally processed (sharpness, brightness and contrast) with ImageJ. **(c)** The reconstituted 3D image of the acquired data in **b**. A View from dorsal side is shown. **(d)** A magnified image of the indicated area in **c**. **(e)** A magnified image of the right hippocampus, viewed from the midline to lateral, as indicated in **c**. **(f)** A magnified image of the reconstituted X-Z image of the right hippocampus, as indicated in **c**. **(g)** The reconstituted 3D whole-organ images from β-actin-nuc-3×mKate2 KI mouse[19] (male, 8-week-old) (imaging condition: Z = 20 μm step × 350-500 planes, zoom = 0.8×-1.6×, expose = 100 ms to 2 s × two illuminations from each side, total acquisition time = about 45 min). The samples were cleared and stained with SYTO 16 according to the CB-Perfusion protocol. The magnified images of SYTO 16, mKate2, and merged signals at the approximate 1 mm depth of each organ (zoom = 5×). **(h)** The reconstituted 3D whole-organ images from a CAG-EGFP Tg[35]

stained with PI (male, 8-week-old) (imaging condition: Z = 20 μm step × 350-500 planes, zoom = 0.8×-1.6×, expose = 100 ms to 2 s × two illuminations from each side, total acquisition time = about 45 min). The samples were cleared and stained with PI according to the CB-Perfusion protocol. Brightness/contrast and minimal gamma-value of images in **c-h** were adjusted with Imaris. All animal experiments here were approved by the Animal Care and Use Committee of the RIKEN Kobe Institute and The University of Tokyo, and all of the animals were cared in accordance with the Institutional Guidelines.

Figure 5

Preprocessing of acquired 3D image for comparison analysis. Here we use the dataset of the Thy1-YPF-H Tg mouse brain acquired in ref. 18 as an example. “Collect raw images” panel shows the scheme of brain 3D imaging of two different directions (D-V and V-D). Raw TIFF images (here only YFP channel shown) in the panel are Z = 2.25 mm (sharp) and 5.5 mm (blurred) for upper and lower panels, respectively. The raw dataset are downscaled to 25% and converted to NIfTI-1 files (shown as capture images of ITK-SNAP). Then, structural D-V data (shown as reconstituted 3D images) via nuclei counterstaining are registered to the corresponding V-D data. This step is to

calculate transformation parameters, which is applied to the signal D-V data in the following step (alignment). We then merge the aligned images in order to ensure sharpness throughout the resulting 3D image (YFP channel is shown as an example again). To do so, the “edge content” based on the Prewitt operator is calculated for both D-V and V-D images. This is used to define two threshold values at Z slice position *n* and *m* and to create the merged composite NIfTI-1 image (shown as capture images of ITK-SNAP) according to these values.

The reconstituted 3D images and plane images in the “Align” and “Combine” panels were prepared by using exported TIFF images from the corresponding NIfTI-1 data. 3D reconstitutions were performed with Imaris software as in **Fig. 4**, and shown as views from dorsal and ventral side in D-V and V-D images, respectively. All animal experiments here were approved by the Animal Care and Use Committee of the RIKEN Kobe Institute and The University of Tokyo, and all of the animals were cared in accordance with the Institutional Guidelines.

Figure 6

Calculation of signal subtraction. **(a)** Here we use the dataset of the Arc-dVenus Tg mouse brains with or without light stimuli, acquired in ref. 18 as an example (Z = 10 μm

step × 625~675 planes, zoom = 2×, expose = 3 s × two illuminations for Venus and 300 ms × two illuminations for PI, respectively). The reconstituted 3D images from raw TIFF stacks are shown as views from dorsal and ventral sides in D-V and V-D images, respectively. Yellow, Venus. Blue, PI. **(b)** We first preprocess these datasets as in **Fig. 5**. Then, we align one dataset, Light (+), to the other, Light (-), by registering the first structural data to the second (internal reference). The internal reference is also registered to a brain atlas such as the Allen Brain Atlas. All images are then aligned to the atlas and normalized. The 3D reconstituted images from NIfTI-1 data for structure and signal of Light (+) or (-) samples, after alignment to the internal reference (step 1 in the panel), are shown as dorsal side view. **(c)** Reconstituted 3D images of aligned and normalized Venus channel (yellow) and aligned PI channel (blue) images from the corresponding NIfTI-1 data. Views from dorsal side are shown. **(d)** Results of subtraction, shown as 3D reconstituted images. Views from dorsal side (left and upper right) and dorsolateral side (lower right) are shown. Signals observed in Light (+) or (-) conditions are shown in magenta and right blue, respectively. Standardized PI signals of Light (+) sample are merged and indicated in blue. As seen in the magnified panel (upper right), single cells in the sparsely labeled regions can be detected even in the downscaled images.

The reconstituted 3D images in **(b)-(d)** were prepared by using exported TIFF images from the corresponding NIfTI-1 data and with Imaris software as in **Fig. 4** and **5**. All animal experiments here were approved by the Animal Care and Use Committee of the RIKEN Kobe Institute, the Gifu University and The University of Tokyo, and all of the animals were cared in accordance with the Institutional Guidelines.

TABLE 1 | Tissues CUBIC has been successfully applied to.

Tissue	Clearing protocol	Reagent before imaging	Clearing efficiency
Mouse whole body*[19]	CB-Perfusion	Reagent-1[††]	Good
Mouse brain[18, 19]	Simple immersion / CB-Perfusion	Reagent-2	Good
Marmoset hemisphere**[18]	Simple immersion	Reagent-2	Good
Mouse heart[19]	Simple immersion / CB-Perfusion	Reagent-2	Good
Mouse lung[19]	Simple immersion / CB-Perfusion	Reagent-2	Good
Mouse spleen[19]	Simple immersion / CB-Perfusion	Reagent-2	Good
Mouse liver[19]	Simple immersion / CB-Perfusion	Reagent-2	Good
Mouse stomach[19]	Simple immersion / CB-Perfusion	Reagent-2	Good
Mouse intestine[19]	Simple immersion / CB-Perfusion	Reagent-2	Good
Mouse kidney[19]	Simple immersion / CB-Perfusion	Reagent-2	Good
Mouse pancreas[19]	Simple immersion / CB-Perfusion	Reagent-2 or Reagent-1	Good
Mouse lymph node[†]	Simple immersion / CB-Perfusion	Reagent-2	Good
Mouse muscle[19]	Simple immersion / CB-Perfusion	Reagent-2	Good
Mouse skin***[19]	Simple immersion / CB-Perfusion	Reagent-2	Good
Mouse bone[19]	CB-Perfusion	Reagent-1	Partially cleared but need further investigations[†††]
Tissues with melanin (eye and hair)[19]	CB-Perfusion	-	Not cleared with the current

reagents

*We performed imaging of postnatal day-1 samples[19]. Imaging of cleared adult mouse was not tested due to size limitation of current LSFM setup.

**So far we tested a brain sample of postnatal day-3. Adult brain will be tested in future studies.

***Need hair removal

†Unpublished result

††The cleared body specimen can be stocked in the reagent.

†††Bone clearing of infant mice was sufficient for imaging[19]

TABLE 2 | Other clearing methods and their applications to imaging and analysis

Methods	**Chemical contents**	**Applied imaging methods**	**Applied computational analysis methods**
CUBIC[18, 19]	Quadrol / Triethanolamine / Triton X-100 / Urea / Sucrose	Ultramicroscope (LSFM), Two-photon microscopy, Confocal microscopy	Whole-brain signal comparison analysis, Extraction of anatomical and histological structures, Quantitative analysis of pancreatic Langerhans islets
3DISCO[4, 5, 7, 36-38]	Ethanol or Methanol / Xylene / Benzyl alcohol / Benzyl benzoate for BABB method Tetrahydrofuran / Dichloromethane / Dibenzyl ether for THF-DBE method	Ultramicroscope (LSFM), Two-photon microscopy, Confocal microscopy	3D reconstruction, Visualization of intensities, Axon tracing, Cell number quantification, Tumor volume calculation
Sca*l*e[9]	Urea / glycerol / Triton X-100	Two-photon microscopy, Confocal microscopy	3D reconstruction, Distance measurement
SeeDB[10]	D(-)-Fructose / α-thioglycerol	Two-photon microscopy, Confocal microscopy	3D reconstruction, Axon and dendrite tracing, Cell distribution analysis

FRUIT[14]	D(-)-Fructose / urea	Two-photon microscopy	
Clear^{T}[11]	Formamide for *Clear*^{T}, Formamide and polyethylene glycol for *Clear*^{T2}	Optical Sectioning Microscopy, Stereomicroscopy	
2,2'-Thiodiethanol[1, 2, 13]	2,2'-Thiodiethanol	Two-photon microscopy, Confocal microscopy, Two-photon serial sectioning tomography	3D reconstruction
CLARITY and related methods*[13, 15-17]	SDS in borate buffer / one of FocusClear™, RIMS (Histodenz or Sorbitol), or 2,2'-Thiodiethanol	Confocal microscopy, Two-photon microscopy, COLM and other custom LSFM	3D reconstruction, Neurite tracing

*Use acrylamide-embedded specimen

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Possible solution
Preparation (REAGENT SETUP)	Possible degradation of chemicals in CUBIC reagents	Too much heating (which causes ammonia odor)	Milder and shorter heating during preparation
	Precipitate in reagent-2	Lower room temperature, particularly in winter	Prepare the reagent before use; heat with microwave for 5-10 s (avoid boiling)

Fixation (Steps 2A(i) and 2B(iii))	Poor PFA perfusion	Insufficient cooling of PBS and PFA	Keep PBS and PFA on ice just before perfusion
		Wrong position of the tip of needle	Make sure that the tip of needle is in the left ventricle of the heart
		Insufficient pressure for perfusion	Make sure perfusion outlet only occurs at the cut in the liver
Clearing (Steps 2A(iv-viii), 2B(v-ix) and 2C(iii))	Poor organ clearing during reagent-1	Alkaline pH of PFA	Adjust pH of PFA to approximately 7.4
		Too much fixation time	Stop post-fixation within 24 h
		Insufficient incubation time, reagent amount or mixing	Incubate longer in reagent-1 (a few days more); Exchange reagent-1 more frequently (every day rather than every 2 d); Shake or rotate appropriately
		Use of an aged animal	Use a younger animal
		Organ-dependent differences in clearing	Use reagent-1 rather than reagent-2 for the final clearing reagent (e.g. pancreas becomes clearer in the reagent-1, see **Fig. 3**)

Excess shrinkage or deformation of cleared organ after reagent-2	Inappropriate concentration of salt whilst treating with 1/2 reagent-2	If shrunk too much, decrease the concentration of PBS in the 1/2 reagent-2 (e.g. mix 1:1:2 of distilled water:PBS:reagent-2 rather than 1:1 mix of PBS:reagent-2); usage of 1/2 reagent-2/water instead of PBS causes swelling
	Incubation at 37°C	Try to incubate samples at room temperature during reagent-2 steps (takes more time)
	Insufficient replacement in 1/2 reagent-2	Sufficiently incubate in the reagent for complete replacement
	Use of an infant/juvenile animal	Organs from an infant/juvenile animal tend to shrink more in reagent-2 and need less incubation time during reagent-1 treatment
	Use of samples prepared in CB-Perfusion	Try simple immersion protocol
Poor organ clearing during reagent-2	Incomplete dissolution or precipitate in reagent-2	Make sure no precipitate exists in the prepared/stocked reagent-2
	Insufficient incubation in reagent-1	Increase reagent-1 treatment time

		Insufficient replacement into reagent-2	Make sure to incubate at 37°C during reagent-2; Use more volume of reagent-2 and exchange the reagent one or two more times
	Ammonia smell during clearing	Degradation of urea	Change the fresh media, and avoid too much heating during preparation of reagents
	Bubbles on inside structures of organs (e.g. brain ventricles) after reagent-2	Insufficient degas	Degas the reagent-2 after preparation; Degas the sample in a limited volume of PBS before the reagent-2 incubation
		Rotation during reagent-2	Incubate in the reagent-2 with gentle shaking rather than rotation
	Tissue damage on freezing	Keeping the sample at -20°C ~ -30°C (which possibly causes growth of water crystals inside the tissue)	Stock the sample in O.C.T. compound at –80°C; Thaw the sample gradually at room temperature
		Insufficient replacement by sucrose solution	Increase incubation time in the sucrose solution (i.e. until organs sink)
Imaging (Step 6)	Floating sample during imaging	Usage of reagent-2 rather	Immerse the sample in oil

	than the immersion oil mix	
Bubble on the surface	Insufficient removal of the reagent-2	Remove excess reagent-2 before immersion into the oil mix; Remove bubbles in the oil with a needle or a tapered forceps
High auto-fluorescence	Insufficient fluorescent signal	Select a bright fluorescent protein (e.g. YFP, Venus) with a strong expression promoter (e.g. CAG); Avoid green channel and use a red fluorescent protein (e.g. mKate2)
	Alkaline pH of PFA	Adjust pH of PFA to approximately 7.4
	Use of an aged animal	Use a younger animal
Weak or non-detectable fluorescence	Insufficient fluorescent signals	Select a bright fluorescent protein with a strong expression promoter as above
	Signal decrease in CB-Perfusion	Use more PFA for perfusion and pause the perfusion procedure after PFA perfusion to increase fixation reaction (3 h~); Try the immersion protocol; Select an animal strain with a bright fluorescent signal

		Temperature during clearing	Higher temperature during clearing may decrease fluorescence signals[8]. Try to incubate at room temperature rather than 37°C. (Note that lower temperature also decreases clearing efficiency)
		Inappropriate setting of microscopy	Check the setting of laser power, filter and exposure time
	Poor Z resolution	Inappropriate setting of light-sheet focus	Adjust the light-sheet focus to the region of interest (in the case of LaVision Ultramicroscope, the width of focused sheet is 1/3-1/4 of an adult mouse hemisphere and thus it is impossible to take images with adjusted focus throughout the brain)
Analysis (Steps 8-15)	Brain appears deformed in the NIfTI-1 file (Step 8)	Wrong scaling	Check the parameter file and ensure that the correct voxel dimensions are given (for the raw TIFF image, in mm)
	Incorrect brain orientation (Step 8)	Different brain position during imaging	Reorient the NIfTI-1 file in ITK-SNAP until it matches your raw TIFF stack. Note the correct orientation (e.g. RPS) and edit the convertTiffFiles.py script accordingly (line 148 for DV-acquired files, line 150 for VD-acquired ones)

	Error message: "No such file or directory"	Wrong folder name or brain ID in the parameter file	Check the parameter file for that step and ensure that all brain IDs are valid and that all folders exist
	Error message: "Command not found"	Missing software	Make sure that all the required tools are installed, that they are accessible from the command line, and that our two C++ programs are compiled

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COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests (see the HTML version of this article for details).

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Author Contributions Statement

H.R.U., E.A.S., K.T., and D.P. designed the study. E.A.S., H.Y., and A.K. performed most of the immersion protocol. K.T. performed most of the CB-Perfusion protocol. D.P. performed most of the computational image analysis. A.K. developed the improved immersion protocol. All authors discussed the results and commented on the manuscript text.

SUPPLEMENTARY INFORMATION

Supplementary Video 1

Spot-counting analysis of hippocampal neurons in Thy1-YFP-H Tg mouse brain shown in **Fig. 4b-f**. Almost all cells are roughly detected as single spots in such a relatively dense region. The analysis was performed with Imaris software. Some of parameters are manually adjusted.

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