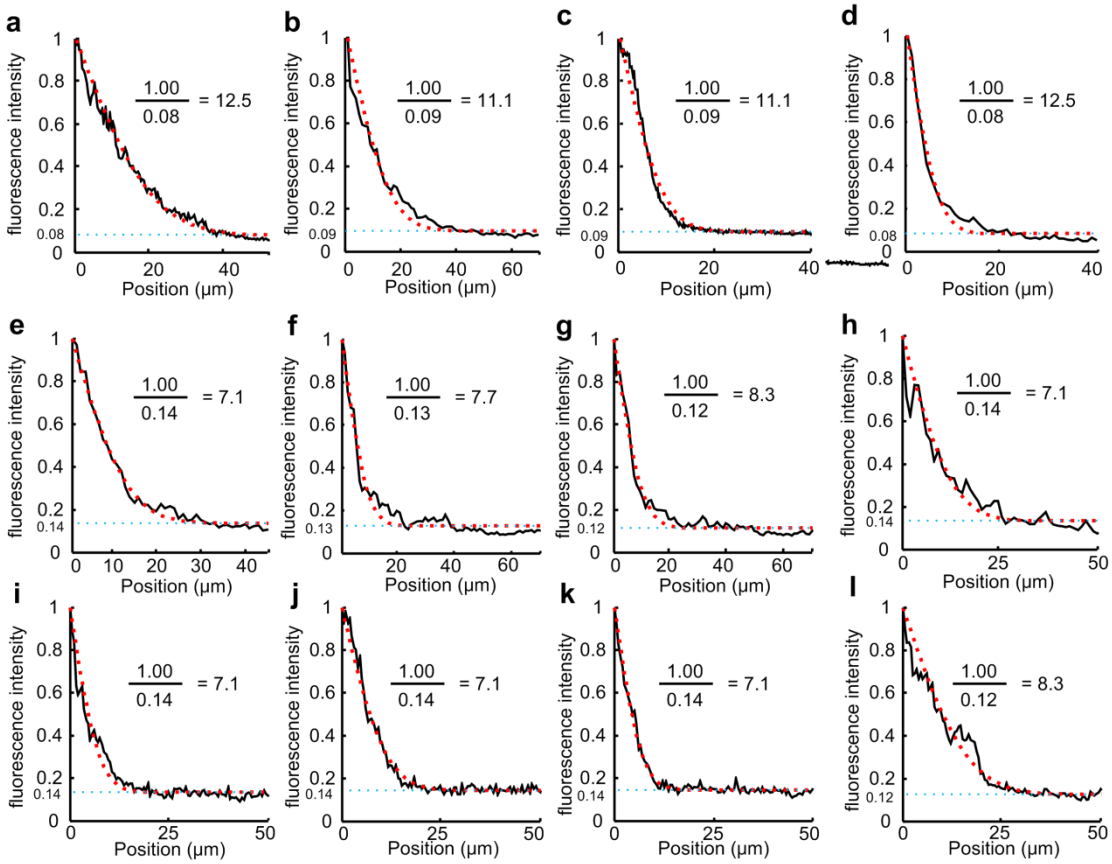
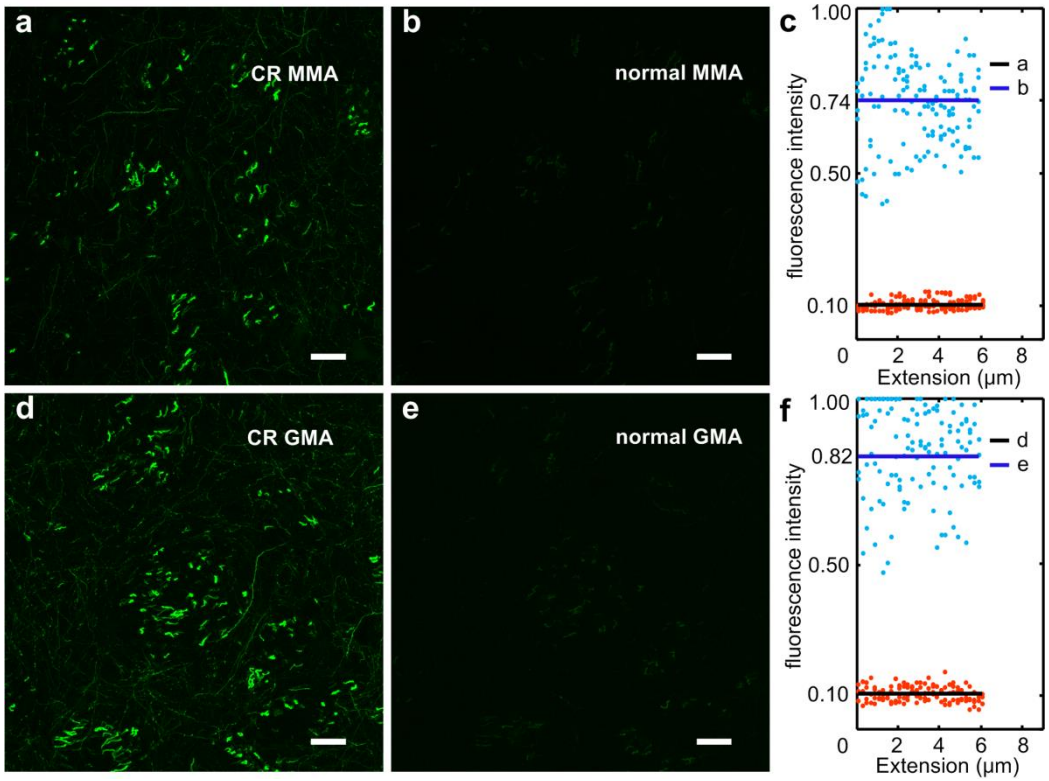
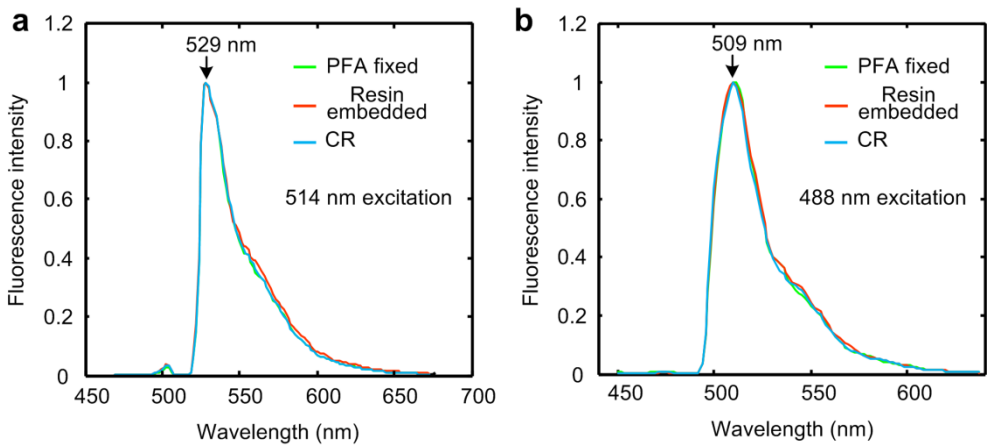
Chemical reactivation of quenched fluorescent protein molecules enables resin-embedded fluorescence micro-imaging



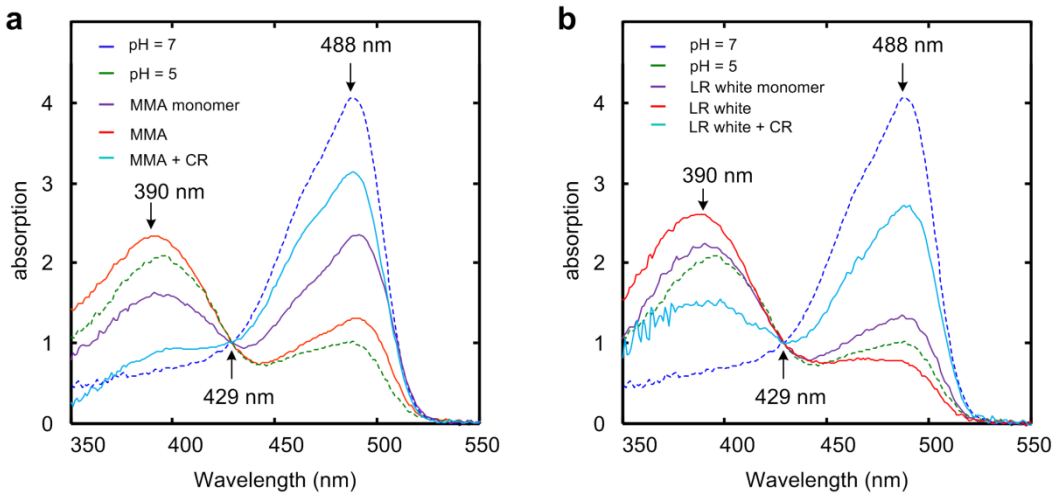
Supplementary Figure 1| Measurements of CR induced fluorescence enhancement on resin embedded tissues. After the resin embedded thy1-EYFP mouse brains were penetrated by alkaline buffer, fluorescence intensity distributions (black curves) along penetration direction were measured, and the distributions were fitted onto the solution of penetration equation (red dash curves) by least square algorithm. (a), (b), (c) and (d) show correspondent results of LR white embedded tissues; (e), (f), (g) and (h) show correspondent results of MMA embedded tissues; (i), (j), (k) and (l) show correspondent results of GMA embedded tissues. Dash blue lines show the Least-squares fitting results of the initial fluorescence intensities before penetration.



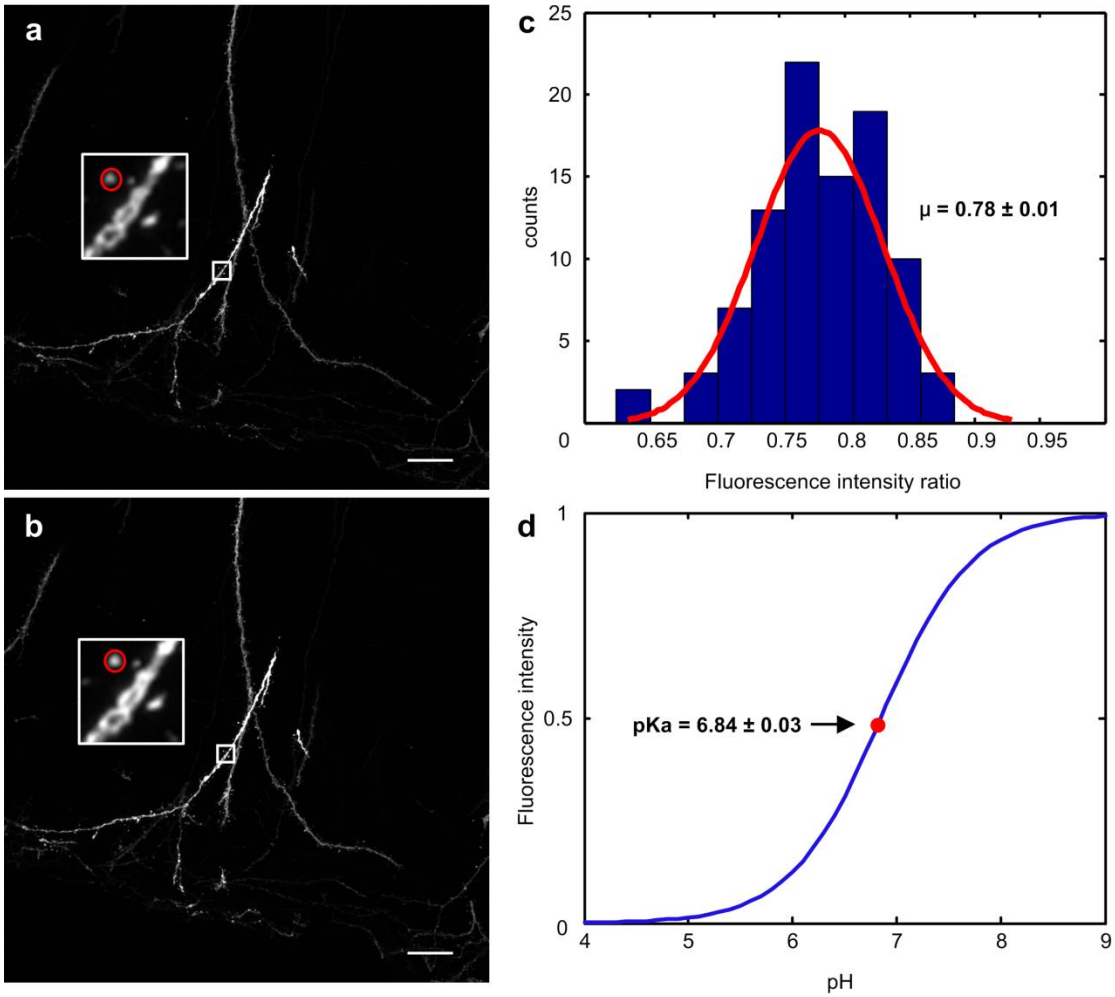
Supplementary Figure 2| Chemical reactivation phenomenon induced on GMA and MMA embedded thy1-YFPH mouse brain tissue. Axial direction extended axons in Striatum regions have been chemical reactivated by alkaline buffer (0.1M Na_2CO_3 solution, pH = 11.6) for two minutes for GMA embedded specimen and ten minutes for MMA embedded specimen separately. Different portions of the axons in MMA embedded specimen: (a) the chemical reactivated portion; (b) uninfluenced portion under the 6 μm chemical reactivated layer. All are maximum intensity projections of 6 μm thick z-stacks. (c) the uniform fluorescence intensities of 4 traced axons. The blue points show the fluorescence intensity of chemical reactivated portion along their extensions; and red points for uninfluenced portion. The solid lines (blue or dark) are the least square fitting results of the fluorescent intensities. scale bar: 20 μm. (d) (e) and (f) is the corresponding figure for GMA embedded specimen.



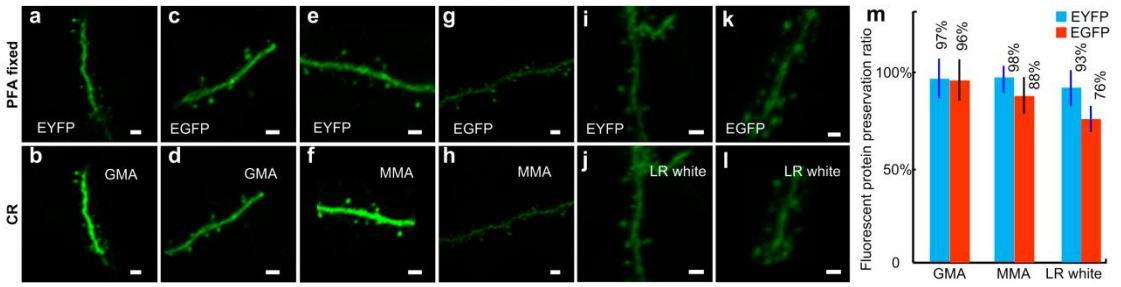
Supplementary Figure 3| Fluorescence spectra of EYFP (a) and EGFP (b) in PFA fixed tissue (green curve), resin embedded tissue (red curve) and CR enhanced resin embedded tissue (blue curve).



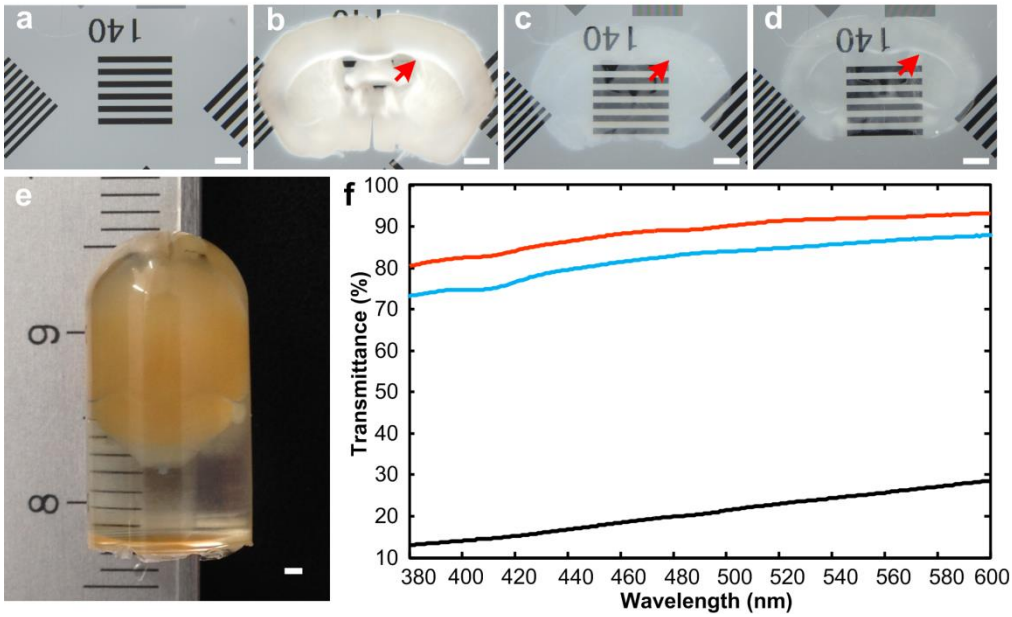
Supplementary Figure 4| Absorption spectra of EGFP measured during resin embedding procedure. (a) For MMA embedding purified recombination EGFP and (b) for LR white embedded one. Blue and green dash lines show absorption of EGFP in distilled water and HAC solution (pH=5), purple lines show absorption of EGFP in resin monomer, red lines show absorption of EGFP in polymerized resin block, light blue line shows absorption of EGFP after CR.



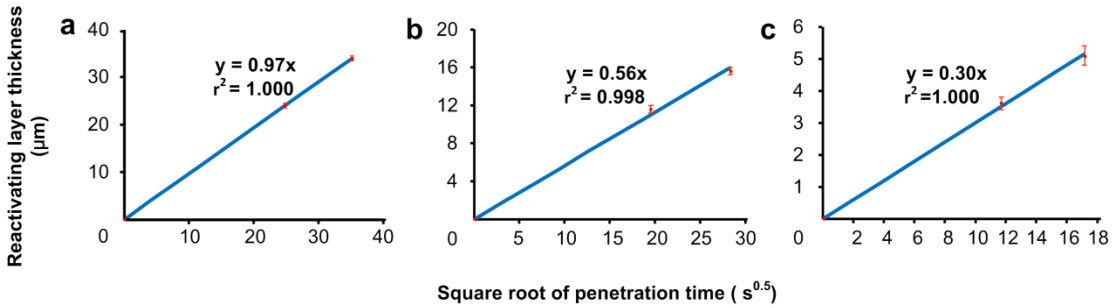
Supplementary Figure 5| The pKa of EYFP in PFA fixed tissue. Apical dendrites on PFA fixed thy1-YFPH mouse brain slice was imaged (a) in PBS (0.01 M, pH = 7.40) and reimaged in (b) pH modified PBS (0.01 M, modified by NaOH, pH = 9.40, the fluorescence intensity reaches the maximum at this pH) with the same microscope configuration. Inserted images in (a) and (b) show the enlargements of the white rectangles labeled areas. (c) The distribution of 94 spines' fluorescence intensity ratio between pH = 7.40 and pH = 9.40 was fitted to a normal distribution. μ represents the estimates of the mean value (within 95% confidence interval). Fluorescence intensities of the spines was the intensity integration on the whole spine body, as present in the red circles in the inserted images in (a) and (b). (d) Titration curve of the EYFP chromophore in PFA fixed state calculated by the measured pKa. The red point show the measured pKa in PFA fixed tissue. scale bar: 20 μm.



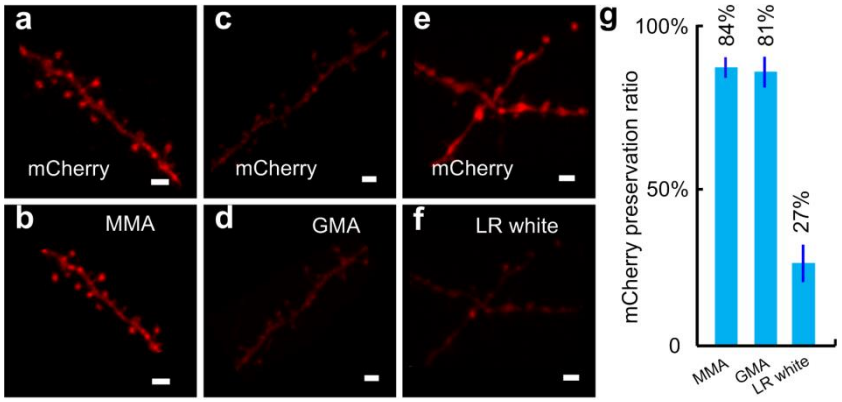
Supplementary Figure 6| Fluorescent protein preservation ratios of CR enhanced resin (GMA, MMA and LR white) embedded fluorescent protein (EYFP or EGFP) labeled tissue. Image of layer V pyramidal neuron dendrites in thy1-YFPM mouse cortex (a) at PFA fixed state and (b) CR enhanced GMA embedded state. During imaging, PFA fixed brain slices were kept in phosphate buffer (0.01 M, pH = 7.4); embedded brain slices were kept in Na_2CO_3 solution (0.1M, pH = 11.6). All images are acquired on the same microscope (Nikon, A1) with same configuration at 25°C room temperature. (c) and (d) show the corresponding figures for GMA embedded EGFP labeled tissue; (e) and (f) show corresponding figures for MMA embedded EYFP labeled tissue; and (g) and (h) show corresponding figures for MMA embedded EGFP labeled tissue; (i) and (j) show corresponding figures for LR white embedded EYFP labeled tissue; and (k) and (l) show corresponding figures for LR white embedded EGFP labeled tissue. (m) Fluorescent protein preservation ratio in resin embedded tissue. Error bars show the standard derivations of six statistic samples. Scale bar: 2μm.



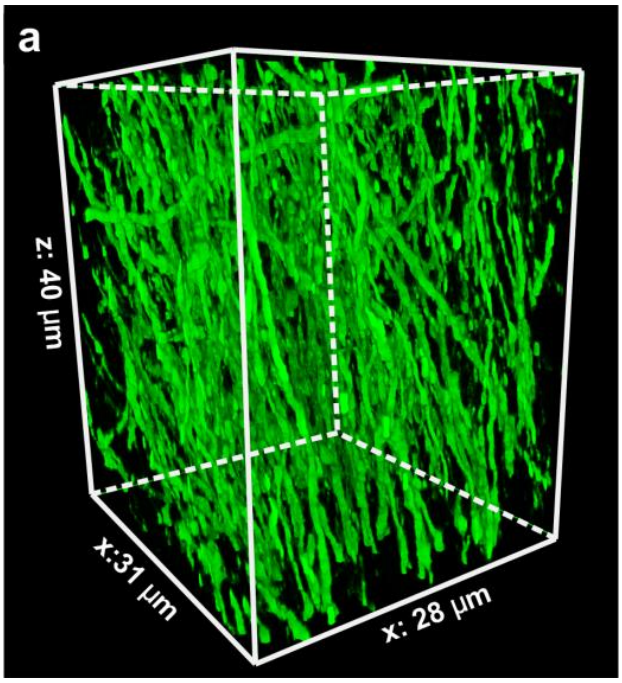
Supplementary Figure 7 | Transparency of the resin embedded tissue. Brain slices were placed on a plate with strips and number marks (a), red arrows show the region of transparency repeatedly changed. Bright field microscopy of a 200 μm brain slice at PFA fixed state (b), MMA embedded state (c) and CR enhanced state (d). (e) A MMA embedded P60 whole mouse brain. (f) Transmittance of 100μm brain slice at PFA fixed state (black curve), MMA embedded state (red curve) and CR enhanced state (blue curve).



Supplementary Figure 8| The relationship between the reactivation layer thickness and the square root of the penetration time. The penetration buffer was 0.1 M Na_2CO_3 solution. (a) For GMA embedded specimen; (b) for LR white embedded specimen; and (c) for MMA embedded specimen. Blue lines show the least square fitting results; red points show the measured results; error bar show the axial direction step size of two photon microscope imaging.



Supplementary Figure 9 | Preservation of mCherry in resin-embedded tissue. Panels (a), (c) and (e) show PFA-fixed mCherry-labeled dendrites. Panels (b), (d) and (f) show the corresponding MMA-, GMA- and LR White-embedded states. (g) mCherry preservation ratios of MMA, GMA and LR White. Error bars show standard deviations based on five particle measurements. Scale bar: 2 μm.



Supplementary Figure 10 | Axon collaterals in a P45 thy1-YFP-H mouse Corpus striatum region. Imaged by CR methods in MMA embedded mouse brain, 60× water-immersion lens, 1.1 NA, with $0.3\times0.3\times0.5\mu m^3$ voxel size.

resin	normal		10 min penetration		Dry after CR	
	block	tissue	block	tissue	block	tissue
LR white	[80, 82]	[75, 77]	[80, 82]	[75, 77]	[80, 82]	[75, 77]
GMA	[82, 84]	[70, 72]	[82, 84]	[70, 72]	[82, 84]	[70, 72]
MMA	[83, 86]	[79, 82]	[83, 86]	[78, 82]	[83, 86]	[79, 82]

Supplementary Table 1 | Hardness of the resin embedded tissue before, during and after CR. The hardness is measured by a Shore D durometer.

Supplementary Discussion 1: The law for penetration of a liquid into a solid block

The mathematic model can be describe like this, for the reign not near the edge of the specimen, the penetration can be considered as a constant concentration buffer (such as n_0) penetration into an infinite wide surface of an infinite thick solid block, which can be simplified to a one dimension penetration processing. Define the $t = 0$ as the time when block was immersed, and defined $N(z,t)$ as the number of molecules that penetrate through the unit area at z point perpendicular to the penetration direction, and $n(z,t)$ as the solution concentration at z point. Propose that the diffusion coefficient D is constant across the block. Then, use the Fick's law, we have,

$$\int_0^t D\frac{\partial n(z,t)}{\partial z}dt = N(z,t) \qquad (1)$$

Since all the molecules beneath z had once penetrated form z, we have,

$$\int_z^{+\infty} n(z,t)dz = N(z,t) \qquad (2)$$

Eliminated $N(z,t)$ from (1), (2), and rewrite the equation by a differential form, we have,

$$D\frac{\partial^2 n(z,t)}{\partial z^2} = \frac{\partial n(z,t)}{\partial t} \qquad (3)$$

The boundary condition can be describe as,

$$n(0,t) = n_0;\ n(z,0) = 0\ (z > 0). \qquad (4)$$

(3) and (4) determined a solution of $n(z,t)$, which can be described as[30],

$$n(z,t) = n_0\left(1 - \frac{2}{\sqrt{\pi}}\int_0^{\frac{z}{2\sqrt{Dt}}} e^{-x^2}dx\right) \qquad (5)$$

The solution described in (5) show that the extension thickness of one specific concentration surface is a linear function of the square root of penetration time, which indicates that, the reactivation layer thickness (the depth of the layer with one specific reactivation ratio, here we use 50%) can also be a linear function of the square root of penetration time. Actually, our measurements of the reactivation layer thickness under different penetration time supports this result (see Supplementary figure 8).

Supplementary Discussion 2: correction inhomogeneous illumination in the coronal atlas

We used the two-photon fluorescence micro-optical sectioning tomography (2P-fMOST) system[29] to acquire the whole brain coronal atlas. The 2P-fMOST system uses the field-of-view stitching strategy to extend the field of view. But for every stitched panel, the illumination cannot be homogeneous, which introduces periodical strips in the raw data. Here we show that we can routinely remove this artifact by simple digital imaging processing method.

The 2P-fMOST scans a horizontal layer of the specimen strip by strip to accomplish the imaging of a millimeter scale coronal plane. See **Figure 1** below. For every strip, the moving stage carries the specimen to move at the y direction; at the same time, the AOD scanner drive the laser beam to scan at the x direction line by line. By this way the 2P-fMOST accomplishes the imaging of a strip. The illumination light intensity is not homogeneous, which varies along the scanning line at x direction, as the diffraction efficiencies of AOD scanner varies at different diffraction angles. The excitation light intensity at the edge of every stripe is always half of the intensity in strip center (see **Figure 1b and c**). This inhomogeneous illumination causes the periodical dark-and-bright strips in the raw coronal section images. The dark region always presents at every stitching region between two strips, and the bright region is always at the center of every strip.

This pattern does not affect the continuity of the neuron imaging, and can be efficiently corrected by off-line digital imaging processing procedures (see **Figure 2**). **References 41** has discussed the algorithm in detail[41]**.** In Figure 4 in the article, the coronal plane used as an inset panel is a corrected image.

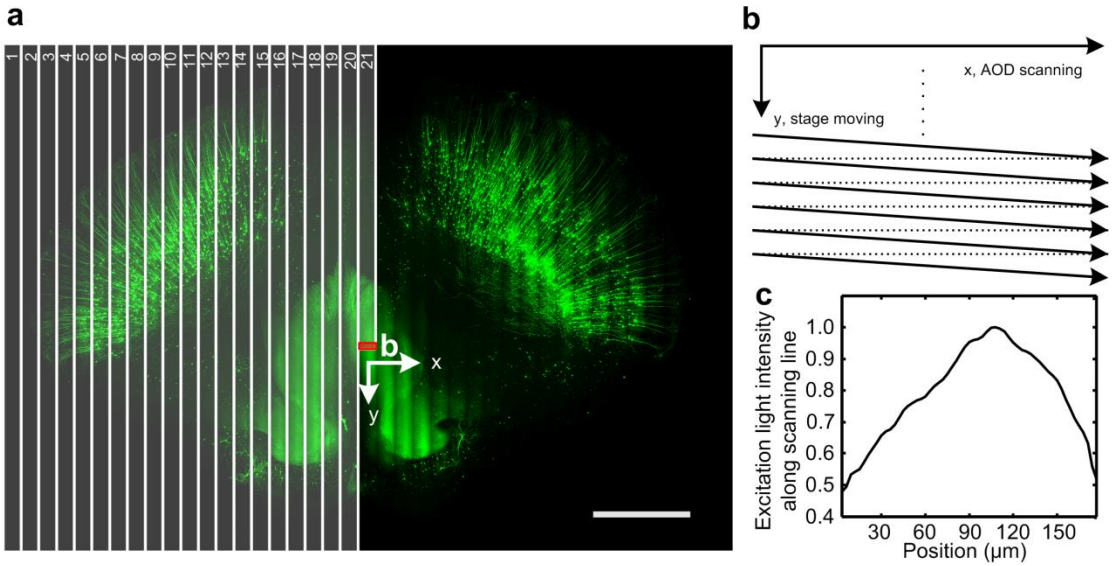


Figure 1 | Scanning strategy of the 2P-fMOST system. (a) Imaging a coronal plane by 2P-fMOST. White box show the imaged strips. (b) The scanning diagram in every strip (such as the red box labeled area in (a)). (c) The excitation light intensity along the scanning line (show as the black lines with arrows in (b)).

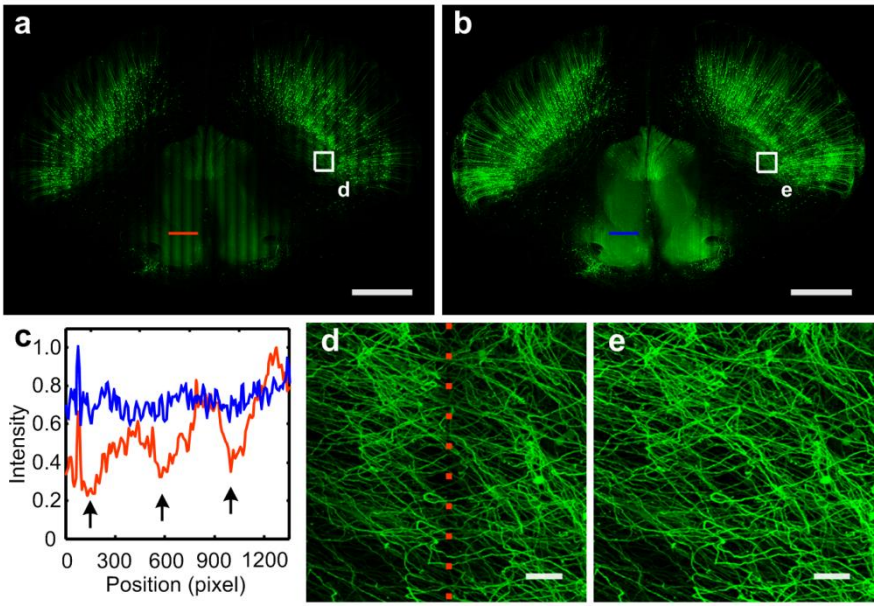


Figure 2 | image corrected by digital imaging processing. (a) The original image with periodical dark-and-bright pattern. (b) The corrected image. Regions labeled by white boxes on (a) and (b) are enlarged in (d) and (e), and gray level of pixels labeled by red and blue lines was plot on (c) with the same colors. Arrows show the stitching positions.

Supplementary Methods

Materials

Samples

Whole mouse brains, P21 Thy1-YFP-H mouse (B6.Cg-Tg(Thy1-YFPH) 2Jrs/J, JAX Mice stock number 003782) and P60 Thy1-EGFP-M mouse (B6.Cg-Tg(Thy1-EGFP) MJrs/J, JAX Mice stock number 007788).

Reagents

Water (distilled)
Ketamine (Fort Dodge)
Phosphate buffered saline (Sigma)
Paraformaldehyde (Sigma)
LR-white resin kit (Structure Probe, Inc.)
Technovit 8100 (a GMA kit, Electron Microscopy Sciences)
Technovit 9100 (a MMA kit, Electron Microscopy Sciences)
Xylene (Sigma)
Ethanol (Sigma)
Na_2CO_3 (sigma)
Cyanoacrylate glue (Fisher Scientific)

Equipment

Constant-current pump (Chuangrui, BT600FJ)
Gelatin capsules (Electron Microscopy Sciences)
Diamond knife (diatome, ultra 35°)
EM trimmer (Leica)

Procedure

Perfusion, Dissection, Post-fixation and rinses

Anesthetize the mouse by intraperitoneal injection of ketamine (5-6 μL/g body weight). After anesthesia, fix the animal's limbs at supine position on a flat board. Then cut into the diaphragm and expose the whole heart. cut the right auricle using a fine scissors and

immediately perfuse 50 mL 0.01 M phosphate buffered saline and then 70 mL neutral buffered paraformaldehyde (4% (w/v) in 0.01 M phosphate buffered saline) into the left ventricle by a constant-current pump at the speed of 0.5ml/min. after this perfusion procedure, cut out the mouse brain using a coarse scissor and then remove the skin tissues and muscles on the mouse head. Then carefully remove the upper skull by small scissors and tweezers and expose the entire surface of the brain. Then the last step for dissection: use a small scissors to cut the cranial nerves, the brain will separate from the rest of the skull. Transfer the whole brain to not less than 20ml neutral buffered paraformaldehyde for 12 hours' post-fixation. After post- fixation, the mouse brain should be transfer into more than 100ml 0.01 M phosphate buffered saline for about 12 hours' rinses.

Embedding

We have tried three frequently used embedding media for CS strategy test: two hydrophilic media, LR-white, technovit 8100; a hydrophobic medium, Technovit 9100. The main embedding protocols followed the product technical data sheets, except some slight modifications and time adjustments.

Dehydration

Successively soaked the tissue through graded series of ethanol aqueous solution on 4 degrees Celsius temperature at dark condition, and for every step, the solution should be more than 20ml per mouse brain. Preparing for hydrophilic and hydrophobic media embedding procedure, the dehydration steps have differences. We separately list them on the followed table:

steps	Solution	Concentration (weight %)	Dehydration time (hour)	
			hydrophilic	hydrophobic
1	ethanol	50	1	2
2	ethanol	70	1	2
3	ethanol	95	1	4
4	ethanol	95	1	-
5	ethanol	Abs.	-	4
6	ethanol	Abs.	-	4

Note: the bar in the table means that the procedure does not have this step.

Intermediate

Only for technovit 9100 embedding do we need an intermediate procedure. The dehydrated brain was soaked into xylene on 4 degrees Celsius temperature at dark condition until the brain tissue is totally transparent.

Infiltration

Here we separately describe the infiltration procedure of the embedding media we have tested. For how to prepare the infiltration solutions, detailed descriptions can be found in the technical data sheets of the embedding medium kits, we will not repeat here. The only trick needs to note here is that the infiltration solution and stock solution A we use was prepared by destabilized basic solution of the technovit 9100 kits.

For technovit 8100 and LR-White embedded specimen preparation, the infiltration procedures are the same, except for infiltration solution different. The dehydrated procedure were implemented by successively soaked the specimen as the steps note below:

steps	Solution concentration (Mixed with 95% ethanol, Weight %)	Infiltration time (hour)
1	70	2
2	85	2
2	Abs.	2
4	Abs.	48

For technovit 9100 embedded specimen preparation, the infiltration steps we used has no difference with the kits data sheets published. The immersing time we use is 2 hour for every groups of pre-infiltration and 48 hours for the last group of infiltration.

After this infiltration procedure, the specimen can be polymerized.

Polymerization

After infiltration procedure, the mouse brain was transfer into the gelatin capsule, add polymerization solution into the capsule until the mouse brain is entirely immersed, and adjust the mouse brain to the proper position using a small tweezers. For LR-White embedded specimen preparation, transfer the capsule into a 55 degrees Celsius oven for about 5 to 7 hours. For technovit 8100 embedded specimen preparation, transfer the

capsule into a 4 degrees Celsius refrigerator for about 12 hours polymerization. For technovit 9100 embedded specimen preparation, firstly drill a hole at the top of the capsule, and then transfer it into a vacuum pump at 4 degrees Celsius for 10 minutes, then transfer it into a -4 degrees Celsius refrigerator, the specimen can be used after about 72 hours polymerization.

Chemical reactivation

When the specimen has polymerized, one end of the block was polished using a rasp, then this polished plane will be stick to a copper specimen holder (Cyanoacrylate glue), and milling the block to expose the plane we are interested by a Milling Machine, at last, this specimen was transferred into a cistern and fixed on the holder by screws. The plane need be finely machined by using a diamond knife. When imaging, we added reactivation buffer (0.01M Na_2CO_3 (pH = 11.2) solution) into the cistern to work as immersion medium for the Water immersion objective (40X 0.75NA). This alkaline solution will immediately penetrate in to the finely machined plane and activate a micrometer level fluorescence intensity enhanced layer for two-photon or confocal microscopy imaging.