Supplementary Information

A. MRI Method

During MRI, the animal was anaesthetized using 3% (+/- 1%) isoflurane in oxygen and received an intraperitoneal injection of sterile saline (3ml) to avoid dehydration during the procedure. Throughout the entire procedure, a mixture of oxygen and 2% (+/- 0.5%) isoflurane was administered to maintain anesthesia. A custom-made head holder (Qualita Ltd., Saitama, Japan) was used to fix the marmoset head within the imaging tube, such that the rostral-caudal axis of the head was stereotaxically aligned with the tube. A small glass capillary with a contrast agent was used in each ear bar, such that the positions of the ear bars would be visible in the MRI images. A heating pad was used to maintain the body temperature. Heart rate, blood oxygen saturation levels, rectal temperature and respiration rate were continuously monitored and recorded every 10 minutes.

During ***in-vivo*** **MR** imaging, high-resolution 3D T1 mapping was carried out using a Magnetization-Prepared Rapid Gradient-Echo (MPRAGE) sequence [1] with a repetition time (TR) = 6000 ms, inversion times (TI) = 150, 1300, 4000 ms, (TE (echo time) = 2 ms TD (time-domain) = 9 ms) and a nominal flip angle (FA) = 12 degrees. Imaging planes were axial slices with FOV = 48.0×38.4×22.6 mm at matrix size = 178×142×42. T2-weighted images (T2WI) were acquired using a rapid acquisition with relaxation enhancement sequence [2] with the following parameters: repetition time/echo time echo = 4000 ms/22.0 ms, RARE factor = 4, averages = 3, field of view = 48 mm × 48 mm, matrix = 178 × 178, slice thickness = 0.54 mm.

Diffusion weighted images were acquired by a pulse-field gradient spin-echo (PGSE, the Stejskal-Tanner diffusion preparation (Stejskal & Tanner, 1965)) based on echo planner imaging sequences along 30 MPG axes and were acquired with the following parameters: b-values = 1000 s/mm^2, TR = 4000 ms, TE = 25.57 ms, averages = 3, k-space segments = 6, matrix = 128 x 128, FOV = 44.8 x 44.8 mm^2, and slice thickness = 0.7 mm. The DTI map was acquired using a method adapted from Fujiyoshi et al. [3]. An eigenvector associated with the largest eigenvalue λ1 was assumed to represent the local fiber direction. Three DTI maps were reconstructed from the data as follows: axial diffusivity (AD) = λ1, radial diffusivity (RD) = (λ2 + λ3)/2, and mean diffusivity (MD) = (λ1 + λ2 + λ3)/3.

For the ***ex-vivo*** **MR** imaging following perfusion, the brain was immersed in an electronic liquid (Fluorinert FC-72; 3M) in a 32 mm ID acrylic tube. High resolution T2-weighted images (T2WI) were acquired using a rapid acquisition with relaxation enhancement sequence [4] with the following parameters: repetition time/echo time echo = 10000 ms/29.36 ms, RARE factor = 4, averages = 16, field of view = 36 mm × 30 mm, matrix = 360 × 300, slice thickness = 0.2 mm. Diffusion weighted image were acquired a pulse-field gradient spin-echo (PGSE, the Stejskal-Tanner diffusion preparation (Stejskal & Tanner, 1965)) based echo planner imaging [5] sequence along 128 MPG axes was acquired with the following parameters: b-values = 1000, 3000 and 5000 s/mm^2, TR = 4000 ms, TE = 28.4

ms, averages = 2, k-space segments = 10, matrix = 190 x 190, FOV = 38.0 x 38.0 mm^2, and slice thickness = 0.2 mm.

B. Tracer Injections

The project plans to cover ~400 injection sites in the marmoset brain, one anterograde and one retrograde tracer at each site, evenly distributed across the gray matter of the right hemisphere of the marmoset brain. The stereotaxic coordinates of all injection sites are systematically chosen using an MRI-based atlas [6]. The choice of the injection location is based on an established algorithm [7]. Briefly, the right hemisphere is separated into 400 equal sized parcels, respecting anatomical boundaries. The plan resulted in 297 injection sites within the cerebral cortex, and 103 injections sites in the subcortical regions. Each subcortical region is evaluated in terms of the structures volume. The injection was then placed based on a grid space modelled for individual structures of interest.

The vector of injection was a borosilicate micropipette with an outer diameter of 20-30µm. At each depth, the tracer was placed at the appropriate depth with an injection speed of 20nl/min. For anterograde tracers, AAVTRE3TdTom (0.3 µl) and AAVTRE3Clover (0.3 µl) were used. For retrograde tracers, Fast Blue (FB, 0.3 µl 5% solution in distilled water; Funakoshi; Tokyo, Japan), Diamidino Yellow (DY, 0.3 µl 2% solution in distilled water; obtained from Prof M. Rosa) and biotin conjugated Cholera toxin subunit B (CTB, 0.6 µl 1% Enzolife, New York, USA) were used.

Post recovery, the animal was housed individually and monitored throughout the incubation period of 4 weeks. The animal received a non-steroidal anti-inflammatory (Oral Metacam; 0.05 mg/kg, Boeringer Ingelheim) for three days immediately following the surgery.

C. Perfusion/Embedding

After the viral incubation time of four week, the animal was euthanized and perfused. The marmoset was injected with Diazepam (Pamlin:2mg/kg) and Ketamine (10mg/kg) followed by pentobarbital (80mg/kg) to anesthetize. The animal was perfused using an 18" oral gavage needle that entered the left ventricle and terminated at the aorta through the aortic valve. 250mL 0.1M PB was used (50ml/min) to remove the blood supply prior to the beginning of asystole to ensure that no clotting occurred; afterwards 500mL of 4% PFA in 0.1M PB was used (70ml/min) for fixation purposes.

After extracting the brain, it was submerged in a solution of 4% PFA overnight. The brain was then transferred to 0.1M PB and underwent a post-mortem *ex-vivo* MRI. Following the *ex-vivo* MR scan, the brain was transferred into 10% sucrose in 0.1M PB overnight and then kept in 30% sucrose in 0.1M PB for means of temperature protection.

A rectangular base mold was custom made with Polylactic Acid (PLA), 3x4x5 mm. A slit was opened from the bottom of the mold, and an additional piece of PLA was cut to fit into

the slit for easy removal of the brain block after the freezing process. A brain mold attached to a positioning bar with its caudal side was also 3D-printed (Figure 2). The brain mold was made from MR images of several marmoset brains [6]. A custom freezing platform, also 3D-printed, secured the base mold flat, and allowed the positioning bar for the brain mold (dorsal side down) to adjust only vertically.

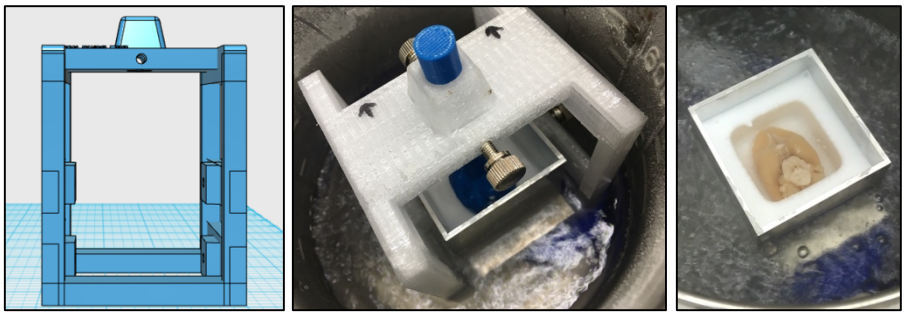

Figure 1. Rectangular base mold was designed and printed to serve as a freezing platform. This freezing platform was used to control the position of the brain model to the base mold during freezing. The positioning bar is adjustable to allow ease of insertion and removal of the base mold from the cast mold.

The brain and the base molds were attached to the freezing platform. By adjusting the positioning bar. The brain mold was lowered such that the dorsal (down) side was 2 mm from the slit. Embedding medium, Neg50 (Richard Allen Scientific, Waltham, MA), was then added into the base mold until it approached, but not immersed, the ventral (down) side of the brain mold. The freezing platform, with the base and brain mold still attached, was situated in dry-ice chilled 2-methylbutane (Sigma Aldrich, 277258) for cryo-embedding.

When the embedding medium was fully frozen, it was thawed by the heat gun to remove the brain mold stick. The surface temperature of the brain mold cavity was -2 ^{o}C to hold the brain shape while leaving the embedding medium a solid. Additional embedding medium was added to the base mold and was left to thermally stabilize for 15 seconds. The brain was removed from the 30% sucrose/0.1M PB solution and dried for 30-45 seconds before being carefully placed within the base mold until it fits inside such that the ventral side of the brain was facing up and it was horizontally at a 0^{o} plane. The embedding mediums volume was sufficient to fully immerse the brain. The base mold was then placed in the dry-ice chilled 2-methylbutane until all embedding medium was uniformly frozen. The base mold was thawed by the heat gun to remove the brain block from the base mold apparatus.

D. Cryo-sectioning

The cryostats stage was modified to accommodate the larger dimensions of the cryo-embedded block and to aid in stabilisation of the cryostats chuck and blade due to the distance of the center of mass from the stage to the cutting plane (Figure 2A and 3B). The UV-LED array was arranged in 4 rows of 11 LEDs in a parallel resistor network to provide uniform UV intensity across the surface of the slides. Each array was connected to a single

6V DC power source and regulated by an on-off timer controlled using a Raspberry Pi [8]. Figure 3C shows the setup of the UV station within the cryostat.

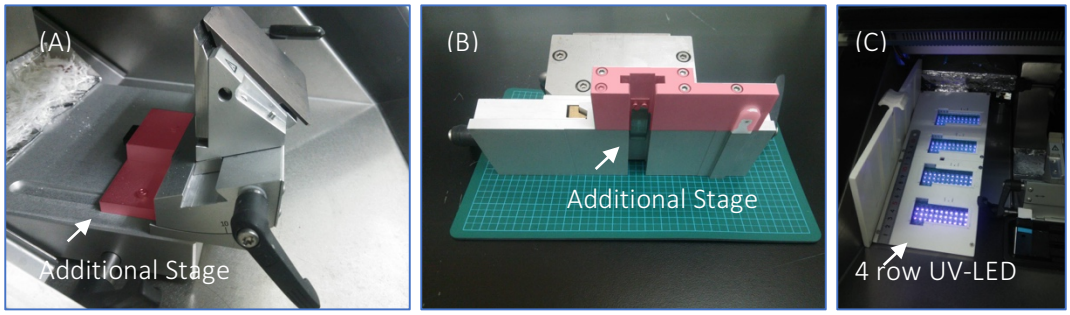

Figure 2. A and B: Additional stage shown in pink was attached to the original cryostats stage to increase the room space and to aid in stabilisation of the cryostats chuck and blade. C: a 4 row UV-LED array to provide UV intensity across the surface of the slides by an on-off timer controlled using a Raspberry Pi.

E. Histology (Staining)

The slides for **Nissl** staining were processed through an automated Nissl staining protocol which began with a thionin solution (1.88g as thionin chloride) in 750mL De-ionized H_2O (DiH_2O), 9mL of glacial acetic acid (WAKO, 012-00245), with 1.08g sodium hydroxide pellets (Sigma-Aldrich, 221465-500G). The slides then underwent three washes of DiH_2O followed by dehydration in increasing concentrations of ethanol 50%, 70%, 95% 100% and finally Xylene. [9,10].

The **myelin** staining technique used a modified ammoniacial silver impregnation technique originally developed by Gallyas [11]. Instead of the standard protocol of implementing the technique on free floating sections, the protocol was applied to the slide mounted sections. After the physical development of the myelin stain, the tissue was manually inspected for staining and morphological quality. The slides were then put on a drying rack for 24 hours and were dehydrated followed by automatic cover-slipping.

The **CTB** designated slides were manually loaded into Immunohistochemistry (IHC) basins (Light Labs, LM920-1). The basins were filled to ½ of their volume with tap water to maintain humidity levels. The CTB-DAB (3,3'diaminobenzidine) protocol had a total of 800uL pipetted onto each slide. The protein block was made of 1%v/v triton X-100 (Sigma Aldrich, X100-500G), 3.5% v/v normal rabbit serum (Vector Labs, S-5000) in 1xPBS for 30 minutes at room temperature (RT), followed by 1xPBS rinse 3 times and pressure assisted drying. The primary antibody step consisted of 2% v/v goat anti-CTB (List Laboratories, #703) (1:5000 concentration), 0.3% v/v triton X-100, 3.5% v/v normal rabbit serum in 1xPBS which was left overnight at RT with the IHC basins covered to preserve liquid levels and ambient humidity within the basin. The secondary antibody was added after 1xPBS rinse 3 times and pressure assisted drying cycle and was made up of 0.4% v/v biotinylated rabbit-anti-goat IgG (H+L) (Vector Labs, BA-5000) (1:200 concentration), 1% v/v normal rabbit serum, 0.3% v/v triton X-100 in 1xPBS for two hours at RT. After 1xPBS wash 3 times and dry cycle the Avidin-biotin complex elite kit (ABC,

Vector Labs, VEC-PK-6100) was placed on the slides and left to incubate for three hours at RT. The ABC kit was used with equal volumes of avidin and biotin. 1% v/v avidin and biotin were made 30 minutes before use.

The DAB-Nickel Cobalt (DAB-NiCo) staining used 1% w/v DAB (Apollo Scientific Limited, BID2042) 1% w/v ammonium nickel (II) sulfate hexahydrate (Santa Cruz Biotechnology, sc-239235), 1% w/v Cobalt (II) Chloride hexahydrate (Sigma Aldrich, 255599-500G) and 0.00003% v/v hydrogen peroxide (WAKO, 081-04215). The DAB, Ni, Co and H_2O_2 were prepared with a DiH_2O in 50mL canonical tubes and filled to 50mL. The 50mL canonical tube which would contain the DAB had 150µl of hydrochloric acid added to make the DAB solution homogenous. 800mL of 1xPBS was prepared and added to an 2L Erlenmeyer flask which was placed on a stir plate, 350µL of 10M NaOH (AppliChem, A3910,1000) was added to bring the final pH of the DAB-NiCo to biological levels (between 7.1-7.4).

The 1% DAB-NiCo solutions were added to the Erlenmeyer flask and homogenized with the aid of a magnetized stir rod. A glass basin large enough to contain 1L of liquid was used inside a fume hood and all slides within the IHC basins were manually loaded into slide racks and placed within the basin. The H_2O_2 was added just before use as it was the catalyst for the DAB-NiCo reaction. The final working solution was transferred from the flask into the basin where the slides had been placed. The incubation time (~10min) was monitored manually until the injection site could be visualized as the affected cells turned black. Manual monitoring was used to make sure that the signal-to-background noise ratio was kept from being deleterious to the final stain quality. The slides were then transferred through 3 full emersion washes of 1xPBS.

The slides were then left on a drying rack overnight at RT and put through Giemsa counterstain (Sigma Aldrich, 12-0440-5-500mL) after a 24-hour period. The Giemsa counterstain consisted of a 3:7 ratio of 30% Giemsa and 70% DiH_2O, a 1xPBS wash, 1% w/v ammonium molybdate wash, a second 1xPBS wash followed with ETOH dehydration. The slides were then cover-slipped and put into drying racks for 24 hours.

F. Computational Infrastructure

All of data machines within the laboratory were connected to data center using a 10g network for further analysis by a 16 node high-performance computing (HPC) cluster. Storage nodes were configured as Raid6 devices and provided 78TB of useable disk space each for a total of 156TB. The theoretical maximum transfer rate of the 10g network is 900 MB/s; however, the rate limiting process was due to the hard disk writing speed of each machine, unless a Solid-State Drive (SSD) was used.

The data processing (cropping and converting) in the data-acquisition server began when the Nanozoomer slide scanning was completed. The processed data were then transferred to a central repository for quality control. This configuration improved the overall process rate from 50% of the theoretical maximum up to 80% in performance. As shown in Figure

3, an entire network and computation pipeline setup was adopted in the RIKEN Marmoset Neural Circuit Architecture Laboratory.

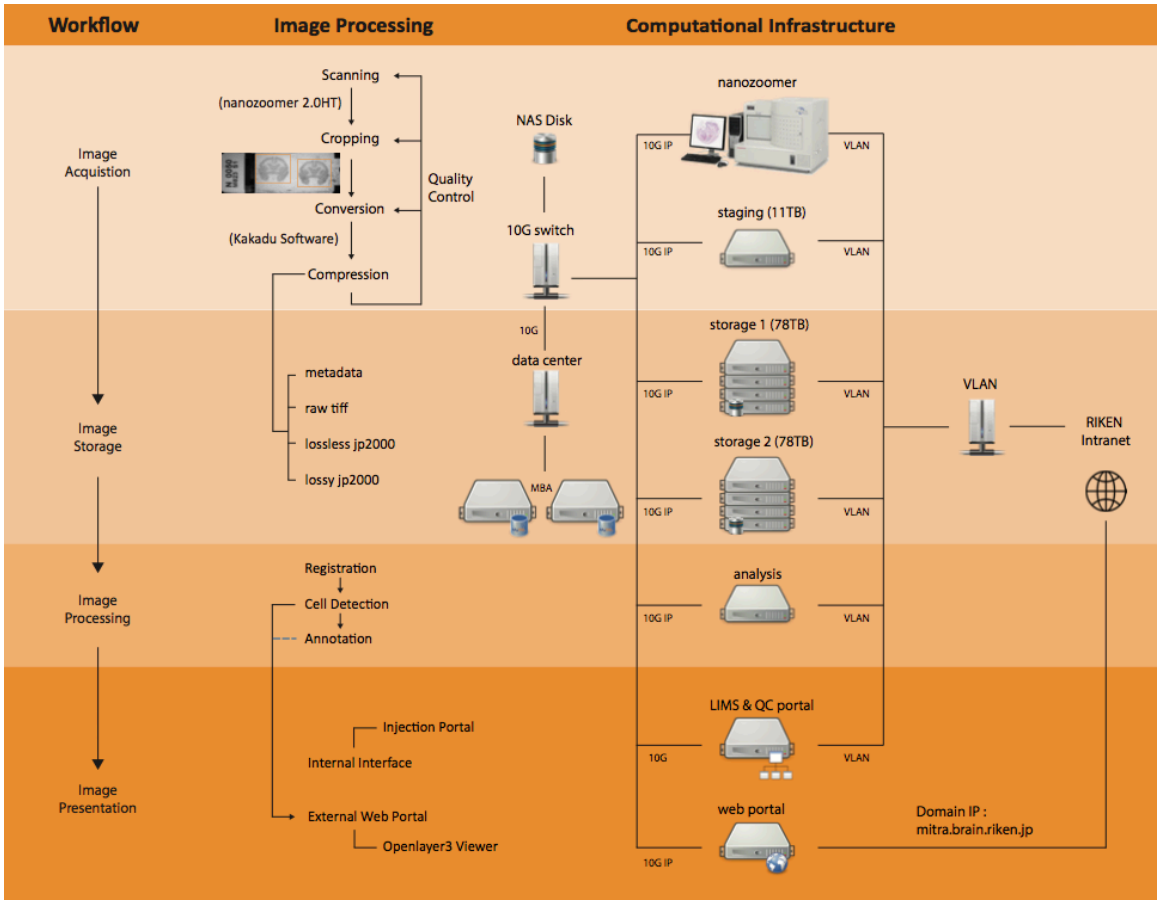

Figure 3. Computational pipeline with the network structure to perform a high-throughput data flow and process. There were four steps of workflow involved in this pipeline including image acquisition, storage, processing and presentation. With these steps, generating a whole marmoset brain dataset with high production rate and superior system performance for large data communication is possible. Each server node was connected to one 10g network for data communication and one external network for remote access.

G. Computational Processing

The computational pipeline is a modified pipeline that builds upon the pipeline originally developed by the CSHL Mitra Lab for the mouse and modified to meet the marmoset tissue size and structure (Figure 4). For each tissue section, the system produces (1) a meta-data file with all the relevant information (cropping and conversion processing); (2) a cropped ROI as a TIF format for image inspection; (3) a down sampled JPEG2000 image for rapid access for the data on the web portal; (4) an uncompressed raw data file. An automatic detection algorithm for the cropping box placement in images performs at a 100% success rate in both the brightfield and fluorescence sections. The image format used custom scripts based on the Kakadu toolkit [12]. For any given complete marmoset brain, there are total ~1700 sections mounted on ~900 slides.

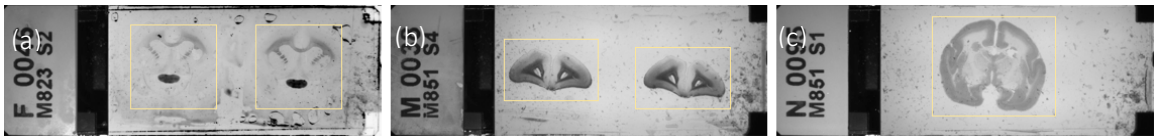

Figure 4. The Nanozoomer macro image determines the cropping ROI (a) fluorescence slide (b and c) brightfield slides shown with yellow cropping box.

This project developed and utilised an online quality control (QC) service. Figure 5 shows the quality control portal displaying all necessary information as well as meta data. The QC service was employed to assess the quality of each image and determine if re-imaging of a slide or the re-injection of an entire brain was needed. Correcting or improving the pipeline process was an evolutionary and organic process and flagging unwanted sections or materials to reduce unnecessary post-processing was a key step. The researcher had the option to view all the sections of the series (with the comparison of a micro image) and to edit the fields for QC such as tissue damage, missing sections and poor cover-slipping alignment. Once flagged, the QC service would automatically remove the sections from the dataset allowing for proper processing of the image analysis such as 2D alignment and 3D reconstruction.

The images of brain slices from histological processing were fed directly into the computational process. The post processing data involved several steps from image cropping and image conversion to 2D alignment and 3D reconstruction. This was a major departure for image analysis. Acquired image datasets were written into a propriety image format, JPEG2000. In the case of a JPEG2000 image, the decompression is ≈ 75-90 MB for fluorescence image brightfield image (Nissl, myelin and CTB). After the proper data acquisition, manual image processing/analysis was performed. The sections across all brains were registered into a common space. This registration was based on an outline of the brain and macro-landmarks such that all sections were able to align to each other and ensure that the features of the images overlap. The 3D reconstruction was performed after perfect re-alignment (transformation of 2D images into a 3D stack).

H. Process Timeline

Based on the individual marmoset brain anterior-posterior length measured by *ex-vivo* MRI, the number of sections (20µm/section) can be determined and the processing time at each step was recorded. At each step of histological processing, a small portion of brain sections were excluded from the subsequent processing based on manual quality control inspection. The final processing success rate for each series was measured by the percentage of obtained sections, shown in Figure 5.

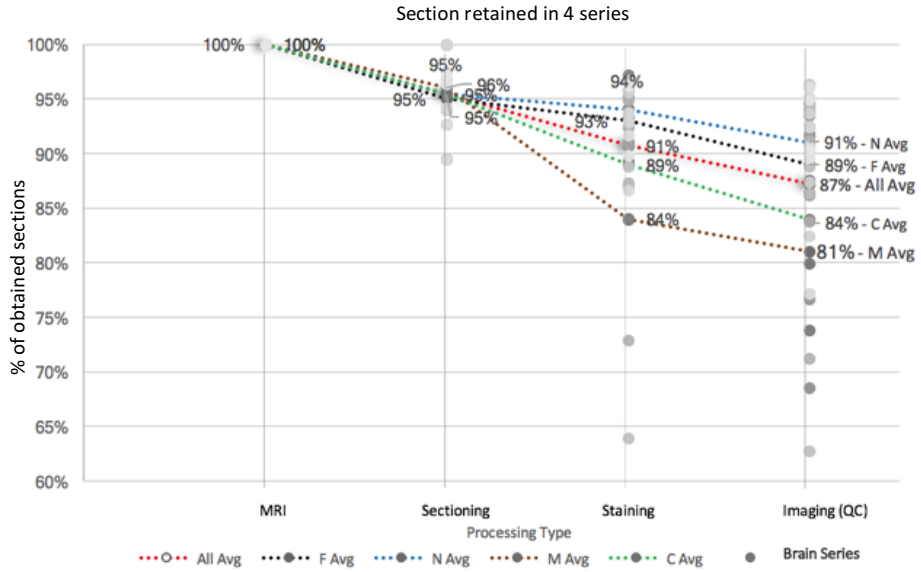

Figure 5. A pipeline processing rate with four series staining (fluorescence, Nissl, myelin, CTB) based on the latest 10 datasets. Each series starts with 100% full rate (based on the calculation from ex-vivo MRI and the number of sections needed as well as calculated by measuring at 20um each) and reduces by a percentage based on unavoidable reasons such as poor staining or section peeling. The figure shows that there is high processing rate starting with Nissl (91%), fluorescence (89%), CTB (84%), to myelin (81%). The average processing rate is 87% in total.

Reference

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