Journal of Visualized Experiments

3D Scanning Technology Bridging Microcircuits and Macroscale Brain Images in 3D Novel Embedding Overlapping Protocol

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58911R3
Full Title:	3D Scanning Technology Bridging Microcircuits and Macroscale Brain Images in 3D Novel Embedding Overlapping Protocol
Keywords:	Brain, neuron, neural networks, MRI, 3D scan, brain mapping, multiscale, connectome, connectomics, connectivity, staining, MEA
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	53 Kawaramachi, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.

TITLE:

3D Scanning Technology Bridging Microcircuits and Macroscale Brain Images in 3D Novel Embedding Overlapping Protocol

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KEYWORDS:

Brain, neural networks, magnetic resonance imaging (MRI), three-dimensional (3D) scan, mapping, scale, connectomics

SUMMARY:

This article introduces an experimental protocol using 3D scanning technology bridging two spatial scales: the macroscopic spatial scale of whole-brain anatomy imaged by MRI at >100 µm and the microscopic spatial scale of neuronal distributions using immunohistochemistry staining and a multielectrode array system and other methods (~10 µm).

ABSTRACT:

The human brain, being a multiscale system, has both macroscopic electrical signals, globally flowing along thick white-matter fiber bundles, and microscopic neuronal spikes, propagating along axons and dendrites. Both scales complement different aspects of human cognitive and behavioral functions. At the macroscopic level, MRI has been the current standard imaging technology, in which the smallest spatial resolution, voxel size, is 0.1–1 mm^3. Also, at the microscopic level, previous physiological studies were aware of nonuniform neuronal architectures within such voxels. This study develops a powerful way to accurately embed microscopic data into a macroscopic map by interfacing biological scientific research with technological advancements in 3D scanning technology. Since 3D scanning technology has mostly been used for engineering and industrial design until now, it is repurposed for the first time to embed microconnectomes into the whole brain while preserving natural spiking in living brain cells. In order to achieve this purpose, first, we constructed a scanning protocol to obtain accurate 3D images from living bio-organisms inherently challenging to image due to moist and reflective surfaces. Second, we trained to keep speed to prevent the degradation of living brain tissue, which is a key factor in retaining better conditions and recording more natural neuronal

spikes from active neurons in the brain tissue. Two cortical surface images, independently extracted from two different imaging modules, namely MRI and 3D scanner surface images, surprisingly show a distance error of only 50 µm as mode value of the histogram. This accuracy is comparable in scale to the microscopic resolution of intercellular distances; also, it is stable among different individual mice. This new protocol, the 3D novel embedding overlapping (3D-NEO) protocol, bridges macroscopic and microscopic levels derived by this integrative protocol and accelerates new scientific findings to study comprehensive connectivity architectures (i.e., microconnectome).

INTRODUCTION:

Nonuniform multiscale architectures at various physical and biological organizations are commonly found[1,2]. The brain is also a very nonuniform and multiscale network organization[3,4]. Various cognitive functions are coded in such network organizations, holding temporal changes of electrical spike patterns of neuronal populations in submillisecond temporal resolutions. Historically, the complex networks among neurons were structurally observed in detail using the staining techniques by Santiago Ramón y Cajal from over 150 years ago[5]. To observe group behaviors of active neurons, researchers have developed various recording technologies[6,7,8], and the recent significant developments of such technologies have enabled us to record electrical activities from huge numbers of neurons simultaneously. Furthermore, from such functional activities, scientists have succeeded to reconstruct networks of causal interactions among huge numbers of neurons and have declared the topological architecture of their complex interactions 'microconnectome'[9]. Macroscopic observations of the brain also allow for regarding a whole brain as a network organization because many brain regions are connected by multiple fiber-bundles. The embedding of microconnectomes into the global brain map still has clear limitations within current technological advances, which is why this embedding protocol is so important. However, there are many challenges to the development of the embedding protocol. For example, in order to observe activities of living local neuronal circuits in purely isolated brain regions, brain slices need to be produced for in vitro recordings. Additionally, recordings from brain slices for in vitro recordings are still an important choice for at least two reasons. First, it is still not easy to observe activities of many living individual neurons simultaneously from brain regions deeper than ~1.5 mm and in high temporal resolution (<1 ms). Second, when we hope to know the internal architecture of a local neuronal circuit, we need to stop all inputs coming from external brain regions to eliminate confounding factors. In order to identify the directions and positions of produced brain slices, it will be further necessary to integrate the spatial positions of these produced brain slices using coordinates. There are, however, a few systematic and reliable ways to make brain slices in an organized way[10,11]. Here, a new coregistration protocol is introduced, using 3D scanning technology for neuroscientific research in order to provide an integrative protocol. This protocol acts to coordinate micro- and macroscales and embed multielectrode array (MEA) microdata[12,13] and staining data onto a macroscopic MRI space through 3D scan surfaces of extracted brains, as well as of noninvasively recorded brains. Surprisingly, this showed a distance error of only ~50 µm as the mode value of the histogram. As a result, the mode values of minimum distances between two surfaces between the MRI surface and the scanned 3D surface were nearly 50 µm for all six mice, which is a suitable number when

checking for commonality among individuals. The typical slice width had a recorded spike activity of around 300 µm.

PROTOCOL:

All experimental procedures described here have been approved by the Kyoto University Animal Care Committee.

1. Animals (day 1)

1.1. Prepare female C57BL/6J mice (*n* = 6, aged 3−5 weeks).

NOTE: This protocol is applicable to all rodent species.

2. MRI settings (day 1)

2.1. Put the mouse in an acrylic anesthesia box placed on a heater mat adjusted to 37 °C using a heater mat controller.

2.2. Anesthetize the mouse with 2% isoflurane in air flowing through the box at a flow rate of 1.4 L/min using an anesthesia system, which is composed of an isoflurane vaporizer, air flow meter, and air compressor.

2.3. After the induction of anesthesia, place the mouse in an MRI-compatible cradle in the prone position. Check if the anesthesia is sufficient by clipping a toe of the mouse with tweezers to see if it does not feel pain.

2.4. Fix the mouse's head with the tooth bar and face mask equipped with the cradle. Then, maintain the anesthesia with 1%−1.5% isoflurane in air at 1.4 L/min via the face mask.

2.5. Insert a thermistor temperature probe into the rectum of the mouse and place a pressure-sensitive respiration sensor on the back of the mouse.

2.6. Transfer the cradle to the bore of an MRI magnet. Adjust the isoflurane concentration to around 1%−1.5% to ensure a stable and reproducible depth of anesthesia. Monitor if body temperature is controlled between 33−35 °C and the respiration rate between 0.5−1.3 Hz during the whole period of MRI experiments, using a monitoring system (**Table of Materials**) for the physiological parameters of small animals with dedicated software (**Table of Materials**).

2.7. Using the measured value of the rectal temperature, regulate the body temperature by controlling the temperature of warm air supplied in the magnet bore from a heater system equipped in the monitoring system.

3. MRI acquisitions (day 1)

3.1. Preparations for the high-resolution 3D T2-weighted MRI scan

3.1.1. Acquire three magnetic resonance (MR) images in three orthogonal (axial, coronal, and sagittal) planes in order to check the mouse's position.

3.1.2. Referring to these images, adjust the position of the mouse by moving the cradle so that the center of the mouse brain is positioned at the center of the resonator as well as at the center of the gradient coils in axial direction.

3.1.3. Adjust the MRI system by tuning the resonator, adjusting the static magnetic field homogeneity (shimming) and the basic frequency, and calibrating the radio frequency (RF) power.

3.1.4. Acquire low-resolution 2D multislice T2-weighted (T2W) images with coronal and sagittal orientations. Based on these images, determine the field of view (FOV) for a high-resolution 3D T2W MRI scan.

3.1.5. Perform localized shimming using a FASTMAP automatic shim algorithm. For the FASTMAP protocol, select a rectangular region that covers the whole brain. After the completion of the localized shimming, confirm the B_0 field inhomogeneity within the brain region by a localized ^1H spectrum by using point-resolved spectroscopy (PRESS).

3.1.6. Acquire low-resolution 3D T2W images to make sure the scan settings for high-resolution 3D T2W MRI are appropriate by checking the appropriate position of the FOV, the absence of wraparound (aliasing) artifacts, and the efficient suppression of fat signals.

3.2. Imaging pulse sequence

3.2.1. After the preparations described above, acquire high-resolution 3D T2W images of the mouse's whole brain, using a rapid acquisition with relaxation enhancement (RARE) sequence.

NOTE: In this article, MRI experiments were conducted on a 7 T, 210 mm horizontal bore, preclinical scanner, equipped with a gradient system of 440 mT/m at ramp time 100 µs. A quadrature volume resonator with an inner diameter of 35 mm was used for RF excitation and signal reception. MRI data were acquired with a dedicated operation software. The acquisition parameters of the 3D RARE pulse sequence used in this study are summarized in **Table 1**.

4. Preparation of experimental solutions (day 2)

4.1. Prepare 500 mL of an ice-cold cutting solution (2.5 mM KCl, 1.25 mM NaH_2PO_4, 7 mM $MgCl_2$, 15 mM glucose, 25 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 11.6 mM sodium ascorbate, 3.1 mM sodium pyruvate, and 100 mM choline chloride, bubbled with 95% O_2 and 5% CO_2). Adjust the pH to 7.3−7.4.

4.2. Prepare 1 L of the artificial cerebrospinal fluid (ACSF; 127 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, 15 mM glucose, 25 mM $NaHCO_3$, and 2 mM $CaCl_2$, bubbled with 95% O_2 and 5% CO_2). Adjust the pH to 7.3–7.4.

5. Preparation of equipment (day 2)

5.1. Prepare a 6.6 cm^2 square, 0.5 cm thick magnet "ring" and, first, place black tape and, second, surgical tape on the ring (**Figure 1c-2**). Note that this material is named a brain-block base (BBB), and keep it cold on blocks of ice.

NOTE: Later, the tapes will be attached to the slicing station with brain blocks for the vibratome. The square ring is used as the bottom base of the BBB to satisfy two purposes, namely to migrate brain blocks smoothly from the 3D scan station to a vibratome and to measure accurately the heights of the brain blocks in the scanning step.

5.2. Set up the BBB with an automatic turntable. Turn on the scanner and the turntable. Start the image processing software.

5.3. Preceding all experiments, perform calibrations of the cameras and the connected turntable of a structured light projection and desktop-type 3D scanner.

5.3.1. Turn on the center projector, the two cameras, and the turntable. Then, open the software. In the software, click **Optical setup** and select the image area as 100 x 80 and the grid numbers as 100 x 100. Then, the camera calibration starts according to the following four steps.

5.3.1.1. For the projector setup, lay the calibration sheet on the desk and put the calibration board on the calibration sheet to adjust the fixing point of the measuring area to the center of the sheet and the orientation to the front. Adjust the distance between the projector and the board to around 200 mm. Loosen the screws fixing the two knobs, and rotate the knobs to adjust the light sensitivity and the focus of the two cameras. Then, click **Arrow** to go to the next step.

5.3.1.2. To orientate the cameras, loosen the screws fixing the two cameras themselves and move the positions and the rotations to overlap with the center line on the calibration board, the vertical line, and the yellow cross projected from the projector. Fix the cameras, and click **Arrow** again. Then, the screen will become dark.

5.3.1.3. To focus the cameras, after loosening the fixing screws of the knobs, increase the light sensitivity and tune the focus again. Then, click **Arrow** to go to the next step.

5.3.1.4. For the F value and the degree of exposure (of the camera), adjust the light sensitivity again to just below where the red region disappears. Then, click **Arrow** again, and click **Calibration start**, and choose **Yes** if asked **Did you finish the optical setting?**

5.3.2. Click **Initialize the calibration**, and check if the pixel values are normalized between 0–255. Then, click **Continue**.

5.3.3. By following suggested positions, record images of nine different relative angles of the scanner and the calibration board, and finish after confirming the calibration process, and, finally, click **Update of the calibration**.

5.3.4. Exchange the calibration board for the turntable, and click the **Turntable calibration**.

NOTE: Now, all calibrations are finished.

6. Brain surface scan, slicing, and MEA recording (day 2)

6.1. Anesthetize a mouse with 1%−1.5% isoflurane and decapitate the mouse. Use a scalpel to open the skin and expose the skull.

6.2. Cut the scalp of the anesthetized mouse along the sagittal suture using a surgical knife, and cut the skull in diagonal directions from the lambda point to a point a little in front of the bregma using surgical scissors. Then, gently peel off the skull from the brain using curved tweezers.

6.3. Use a spatula to lift the brain and transfer it to a Petri dish (100 mm x 20 mm) with ice-cold cutting solution (prepared in step 4.1). Flow air containing 95% O_2 and 5% CO_2 from an external gas bomb with a controlled rotating speed of the pump (~4.0 rpm) to generate small bubbles in the ice-cold cutting solution (**Figure 1a**).

6.4. After 1–2 min, put the brain on a microfiber cloth whose surface is slightly covered by a flour sieve, and wipe fluid from the brain surface, gently using the microfiber cloth (**Figure 1b**).

6.5. Put the brain with its dorsal aspect up on the sample stand on the BBB, and also put the BBB at the center of the automatic turntable.

6.6. Darken the room during the 3D scan and perform a 3D scan on the turntable. To test whether the 3D scan works well, click **3D scan** by selecting the angle between two shots as 22.5, the starting angle as 0, and the final angle as 360 (**Figure 1c-1**).

6.7. Move the brain to a Petri dish, and bubble the brain in the cutting solution for approximately 10 s.

6.8. Cut the brain into two blocks at the middle of the coronal plane using a surgical knife, and move the two brain blocks gently to the flat surface using a surgical spatula.

6.9. Attach the brain blocks of the BBB using instant glue, and softly and carefully wipe the fluid from the brain surface within 1–2 min, using a microfiber cloth again. Then, perform a 3D scan again (**Figure 1c-2**).

6.10. Attach the black tape covering the center of the BBB to the center of the cutting stage for a vibratome (**Figure 1d**).

6.11. Pour cutting solution into the cutting stage and set the cutting stage on the vibratome. Adjust the cutting speed and amplitude. Make 2–5 coronal slices (300 µm thick) from the two brain blocks. Keep the solution in the cutting stage bubbling if possible (**Figure 1e**).

6.12. Attach a blade to the blade holder of the vibratome and optimize the cutting speed, frequency, and vibration amplitude of the system by setting them as 12.7 mm/min for the speed, 87–88 Hz for the frequency, and 0.8–1.0 mm for the swing width. When careful cutting is necessary, slow down the speed to level 2–3.

6.13. While cutting the brain slices, record the sliced coordinate in the format, including the anterior-posterior coordinate, hemisphere, and other conditions (**Figure 2c**).

6.14. Gently transfer the brain slices to a beaker filled with prewarmed (~34 °C) ACSF using a thick plastic pipette, and incubate them in the beaker at ~34 °C for ~1 h. During this time, perform a 3D scan of the remaining brain blocks on the cutting stage (**Figure 1c-2**).

6.15. Set an MEA chip on a recording unit. Connect the chip to a peristaltic pump using two tubes. Use one tube to guide the same ACSF into the MEA chip and the other tube to guide ACSF out of the MEA chip.

6.16. Attach two needles (0.60 mm x 19 mm), connected at the tips of the two tubes, to the top of the wall of the MEA chip, and fix their positions with their tips following the inner wall of the MEA chip. Set the flow rate of the ACSF to 4.1 rpm.

6.17. After 1 h has passed, move a slice on the chip using a thick plastic pipette, and set the position using a soft brush. Then, start the recording process of neuronal activities (**Figure 2-d**).

7. Immunohistochemistry staining (days 3 and 4)

7.1. After the MEA recording is finished, transfer the slices to 1.5 mL tubes filled with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Incubate them overnight at 4 °C. Then, remove the 4% PFA solution, and wash the slices 3x with PBS for 5 min in total.

NOTE: If necessary, embed the slices in 20% gelatin/PBS and resection the slice to make it thinner than 50 µm, using the vibratome.

7.2. Prepare antigen retrieval solution (10 mM sodium citrate, pH 6.0). Remove the PBS from the last wash and add the antigen retrieval solution.

7.3. Boil water in an electric thermos pot and incubate the tubes with the slices for 20 min at ~95–98 °C in the pot, followed by natural cooling.

7.4. Prepare 50 mM Tris-buffered saline and add 1% Na bisulfite (1% Na bisulfite-Tris solution). Adjust the pH to 7.5. Then, wash the slices with 1% Na bisulfite-Tris solution for 5 min at room temperature (~20–25 °C).

7.5. Prepare blocking solution by adding 4% normal goat serum and 0.5% octylphenol ethoxylate to the 1% Na bisulfite-Tris solution.

7.6. Remove the 1% Na bisulfite-Tris solution from the tubes with the slices and add blocking solution. Incubate for 2 h at room temperature.

7.7. Prepare the primary antibody solution by adding 1% normal goat serum and 0.5% Triton X-100 to the 1% Na bisulfite-Tris solution and diluting primary antibodies. Use the following concentrations of primary antibodies: 1:800 of mouse anti-GAD67 and 1:500 of rabbit anti-NeuN.

7.8. Remove the 1% Na bisulfite-Tris solution from the tubes with the slices and add the primary antibody solution. Incubate it overnight at 4 °C.

7.9. Prepare 50 mM Tris-buffered saline and add 8.5 g/L NaCl (Tris-NaCl solution). Titrate the pH to 7.5. Then, wash the slices 3x with the Tris-NaCl solution (~20–25 °C) for 5 min in total.

7.10. Prepare the second antibody solution by adding 3% normal goat serum and 0.3% Triton X-100 to the Tris-NaCl solution and diluting secondary antibodies. Use the following concentrations of secondary antibodies: 1:500 of goat anti-mouse and 1:500 of goat anti-rabbit.

7.11. Remove the Tris-NaCl solution from the tubes with the slices and add the secondary antibody solution. Incubate it for 2 h at room temperature. Then, wash the slices 3x with the Tris-NaCl solution for 5 min in total.

7.12. Place the slices on the slide using a small brush and apply mounting media and a coverslip. Examine the slides with a confocal microscope. Take images of the surface of a slice (**Figure 2e**).

8. MRI data processing to extract cortical volumes

8.1. Install the free software FSL (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FslInstallation). Prepare the MRI images acquired in section 3.

8.2. Change the image format using the **fslchfiletype NIFTI_PAIR_GZ** command. Expand the brain image to make it as big as a human brain using the **fslcreatehd** command, and, if necessary, change the orientation using **fslorient** with the option **-setqformcode 1**. After this, recover the original file format using **fslchfiletype NIFTI_GZ**. The exact command is as follows.

fslchfiletype NFTI_PAIR [input image].nii.gz
fslcreatehd 144 196 160 1 0.95 0.6 1.15 0 0 0 0 4 [input image].hdr.gz
fslorient –setqformmcode 1 [input image].hdr.gz
fslchfiletype NIFTI_GZ [input image]

8.3. Type **fsl** to open the FSL software. Extract brains using the **bet** command from the graphical user interface (GUI), but do not peel too much. Then, control the **Fractional intensity thresholds, ...** and **Coordinates of centre of initial brain surface sphere** carefully. The optimal values are different for individual data.

8.4. Resize the brain image to the original mouse brain size, using the same procedure as in step 8.2. The exact command is as follow:

fslchfiletype NFTI_PAIR [input image].nii.gz
fslcreatehd 144 196 180 1 0.1 0.1 0.08 0 0 0 0 4 [input image].hdr.gz
fslorient –setqformmcode 1 [input image].hdr.gz
fslchfiletype NIFTI_GZ [input image]

8.5. Coregistrate all brain images using the **flirt** command, and produce an averaged brain image using the **-add** option and the **-div** option of the **fslmaths** command.

fslmaths [brain image 1] –add … –add [brain image N] –div N [averaged image name] –odt float

8.6. Make the averaged brain cleaner using the **-thr** option of the **fslmaths** command. Then, select an optimal threshold value adaptive to individual cases.

fslmaths [averaged image name] –thr [thr value, for example, 5000] [thresholded average image]

8.7. Finally, reshape the averaged brain image into an individual's coordinate using the **flirt** command again. Then, prepare fairly clean extracted cortexes (**Figure 2a**).

NOTE: Unfortunately, the olfactory bulb and cerebellum will not be perfect in the reconstructed brain surface from the MRI volumes because of the FSL software's essential limitations to peal all brain parts including olfactory bulb and cerebellum.

9. MRI image processing to stripe cortical surfaces

9.1. Download another free software, 3D Slicer (https://download.slicer.org/).

9.2. Open the MR images of the extracted brains (click **File** and select **Add data**) produced according to section 8, using the Volume Rendering and Editor modules in 3D Slicer.

9.3. Change the mode from **Editor** to **Volume rendering**, and click a target button to make the image of the brain come to the center of the screen.

9.4. Select the **MR-T2-brain** mode and tune the threshold by moving the **Shift** bar. Then, move back from **Volume rendering** to **Editor** and click the **Threshold effect** button.

9.5. Apply the Label **41. Cerebral cortex** and save the brain surface data as a **.stl** file. Then, change the file format from **.vtk** to **.stl**, and save the file by checking the form.

10. Preprocessing for 3D scan data

10.1. Perform an automatic coregistration among 8 or 16 images taken from 8 or 16 different angles within a sequence of a scan to correct small mismatches among them by clicking **Global registration** included in the **Alignment** option, and integrate them. Repeat scans from different angles to obtain the whole-brain surface (**Figure 2b**).

10.1.1. If the integration among images scanned from different angles was not successful, click **Manual alignment** and select a pair of a **Fixed image** and a **Moving image**.

10.1.2. Click **Start** to start the manual alignment of the images by selecting three or four common points in different images. Then, click **OK**. The optimization algorithm is the iterative closest point (ICP) algorithm[10]. It does not include a nonlinear deformation.

10.2. Make a mesh of all the aligned images (i.e., of the dots data sets) by selecting all images and by clicking the **Mesh generation** button. Then, select the option **Small artistic object** to get the mesh as the highest resolution. Then, save the image as .stl binary, or in ASCII format.

11. Coregistration of the MRI surface and the 3D scan surface

11.1. Open the MRI surface and merge it with the 3D scan surface using the surface processing software. Then, perform a manual alignment process using the same procedure as described in steps 10.1.1 and 10.1.2. Then, save these coregistered surface images again (**Figure 2a,b**).

11.1.1. If necessary, clean the individual surface data, for example, by erasing small noises surrounding the brain region, especially in the case of the MRI data, and by filling holes if any exist, especially in the case of the 3D scan data.

11.2. Finally, open the surface data with a data analysis software, for example, MATLAB, and perform and evaluate the histograms of the minimum distances between the two surfaces.

REPRESENTATIVE RESULTS:

We evaluated distances between cortical surfaces, produced by stripping MRI volume, and surfaces obtained from 3D scans of extracted brains. The mode values of the histogram of the distances are only 55 µm (**Figure 3a**). Additionally, when accumulating the histogram from the point where the distance equals zero, the accumulated value reaches 90% of total sample numbers at ~300 µm (**Figure 3b**). The final histogram of the distances between two surfaces

showed a typical peak around 50 µm. If we interpret this value from a macroscopic viewpoint, interestingly, the accuracy, the mode value ~50 µm, corresponds with the geometrical limitation, which was expected from the voxel size of MRIs (**Figure 3a**), namely, 100 µm. This point indirectly suggests that the overlapping algorithm, ICP, between the MRI and 3D scan worked superbly well and that the noise levels of both the MRI and the 3D scan were suppressed as low value (**Figure 2a,b**)[14].

FIGURE AND TABLE LEGENDS:

Figure 1: The experimental flow. (**a**) First, after extracting a brain from a mouse, the brain was dropped into a bubbled cutting solution. (**b**) After wiping the cutting solution off with a soft and highly absorbent towel, (**c**) the brain was scanned on a turntable. (**d**) In making slices (step 8 of the process), the brain blocks were scanned on a BBB because it is easy to move brain blocks to the base for a vibratome (steps 7 and 8 of the process). (**e**) After getting enough slices for recording electrical activities or for staining the cell distributions and other methods, the remaining brain blocks were scanned again (step 10 of the process). The thickness of the remaining brain blocks provided additional important support information of where the slices were taken from. (**f**) Finally, we recorded the functional activities or structural architectures using MEA or staining methods and other methods.

Figure 2: The data processing pipeline. (a) An example of a cortical surface produced by stripping cortexes from MRI volumes as explained in sections 8 and 9 of the protocol. (**b**) An example of a cortical surface directly scanned by a 3D scanner system. (**c**) An example of a memo used to summarize the brain region from where the individual brain slices were extracted. (**d**) An example of when the cortical slice is on an electrode dish. (**e**) An example of a stained brain cortical slice by NeuN (red) and GAD67 (green). All information is now going to be gathered in a unified database.

Figure 3: Overlapping accuracy between MRIs and 3D scans. (**a**) Histograms of distances between surfaces extracted by peeling from MRI volumes. The surfaces came from 3D scan recordings. The main bar graph is the averaged histogram for all individuals, and other colored lines are results for individual mice (*N* = 6, aged 21–40 days, all were female mice). A general trend, stable for all individuals, could be found. (**b**) The accumulated value of the histogram is shown as a bar graph. The accumulated percentage reached 90% at ~300 µm (dotted lines).

Figure 4: Conceptualized image of two scales of information integrated into one graphical user interface (GUI). Both the macroscopic brain anatomy and the microscopic neuronal circuit information were integrated into one system represented by a web. The high degree of accuracy, shown in **Figure 3**, enabled us to integrate these two scales realistically. The macroscopic image presented here is from a scalable brain atlas[15].

Table 1: The acquisition parameters of the 3D RARE pulse sequence used in this study.

DISCUSSION:

We developed a new protocol called the 3D-NEO protocol to bridge macroscopic and microscopic spatial scales by overlapping two brain surfaces more accurately than before. Originally, there were two challenges in creating this protocol which made possible the accurate overlapping of two brain surface images and recording healthy neuronal activities from living organisms. First, it was necessary to effectively wipe cutting solution surrounding the extracted brain after extracting it from the skull without injuring the brain organism (step 6.2 of the protocol). Second, since the first and third conditions of dryness and time delay are potential negative factors for the living organism, finishing all the steps of the scanning process from brain extraction to the slice preparations within 10–15 min was also necessary to keep the neurons active.

The ability to successfully record brain activities using this protocol has now made possible the coordination not only of structural organizations but also of functional activities on the whole-brain map. Another positive aspect of this protocol is that since a BBB was prepared, the calibration from the scanner to the base of the vibratome became much smoother.

As mentioned in the representative results, the histogram of distances between two surfaces showed a peak at around 50 µm. Although we performed the overlapping process, as if by seeing from the macroscopic viewpoint. If we interpret the result from the microscopic viewpoint, the 50 µm is a comparable spatial scale in the distribution of neurons, since connectivity probabilities between pairs of cortical neurons decay within ~100 µm[16,17], even within several millimeters[18]. Practically, MEA or calcium imaging studies often use 300–400 µm-thick slices. So, the technique presented here will provide strong objective evidence if the slices are properly embedded into original whole-brain maps. After overlapping is accurately achieved, additional accuracy will be gained purely from the local information observed under a microscope. Therefore, by performing a two-step optimization process consisting of global and local optimization steps, it will be possible to reach a spatial resolution equating semi-automatically to the typical size of a neuron in the future.

This accurate integration protocol provides basic technology to generate various brain atlases[18,20,21] and even to study more in-depth the MRI of other primates[22,23] and human postmortem brains (although the human brain has sulci, it is possible to get a sufficient number of recording points from gyri). Now, we are also developing a visual interface to integrate many recorded data samples into one common spatial coordinate. One image figure such as this is shown in **Figure 4**. In the cases of mammalian brains, the integration between quantitatively different scales will also make new advances qualitatively in understanding disease states and in evaluating drug effects. Generally, this will be hard to apply to fishes, reptiles, and amphibians, because researchers will find it difficult to keep the form of the extracted thin brain stable to its pre-extracted form, and MRI images of their smaller brains will also be relatively noisier.

The 3D-NEO protocol has been introduced by this research into a neuroscientific or cell biological research field, successfully demonstrating high accuracy in bridging between macroscopic anatomy and microscopic neuronal distribution, including the topological architectures of macro- and microconnectomes.

ACKNOWLEDGMENTS:

M.S. is grateful for the support from all of the staff in the medical information engineering course in Graduate School of Medicine and Faculty of Medicine, and wants to thank Prof. Tetsuya Takakuwa, Prof. Nobukatsu Sawamoto, and Doris Zakian for their helpful comments. This study was supported by a Grant-in-Aid for Challenging Exploratory Research and by the Leading Initiative for Excellent Young Researchers (LEADER) program to M.S. from MEXT (The Ministry of Education, Culture, Sports, Science, and Technology). The MRI experiments in this work were performed in the Division for Small Animal MRI, Medical Research Support Center, Graduate School of Medicine, Kyoto University, Japan.

DISCLOSURES:

The authors have nothing to disclose.

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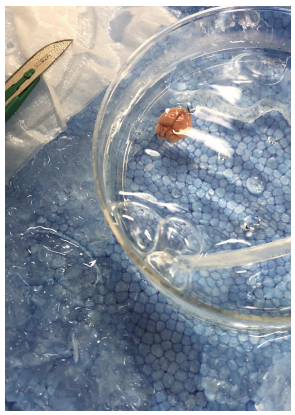
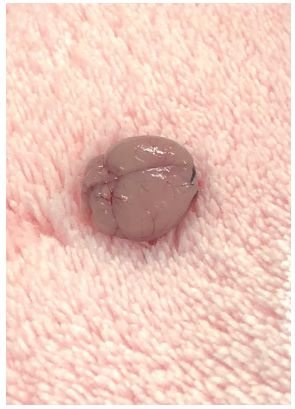


(a)

Bubbling

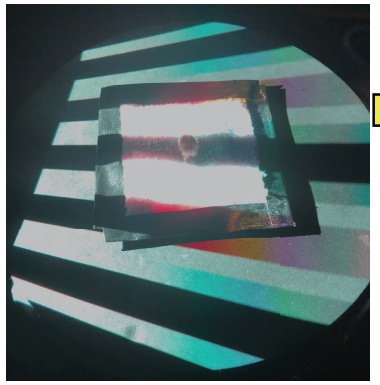
1)



(b)

Wiping

2)



(c)-1

Scan

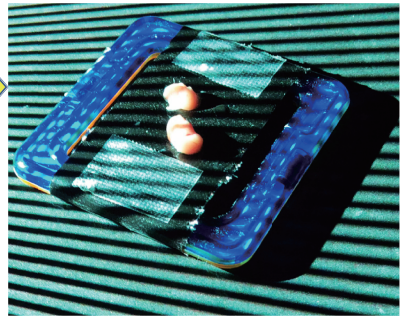
3)

Upside-down
& scan again

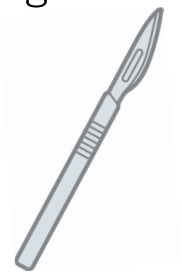
4)

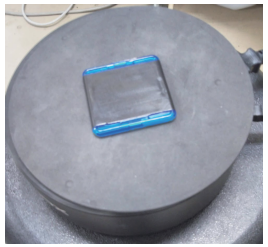
5)

6)



(c)-2





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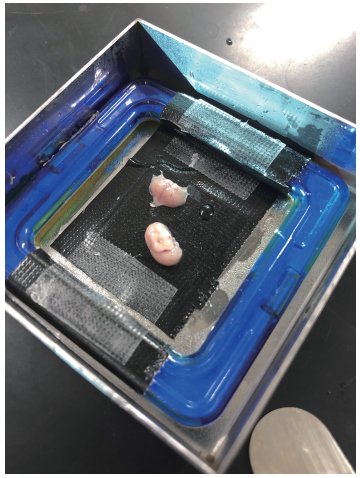
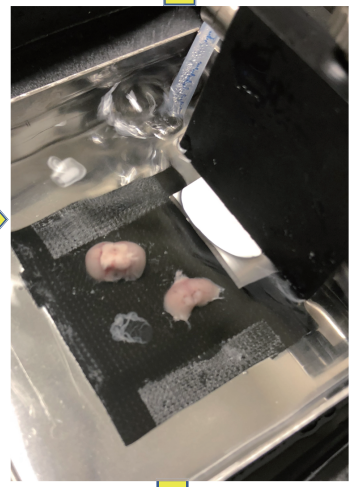


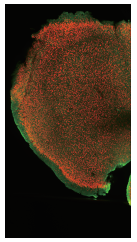
(d)

8)



(e) Slicing

9)



(f) Structural and functional
Recordings from neurons

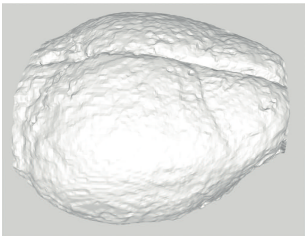
10)

Remain

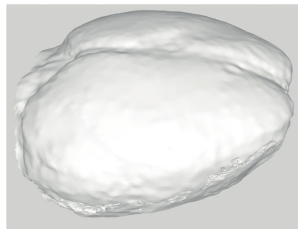
Figure2

Click here to access/download;Figure;figure2.

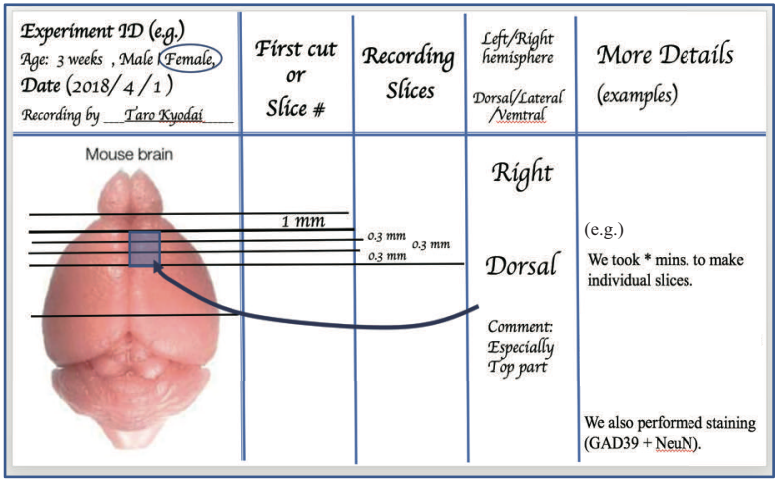
(a) Striped MRI



(b) Connected 3D scan

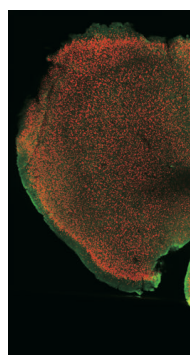


(c) Slicing with experimental record



(d) Neuronal recording

(e) Staining



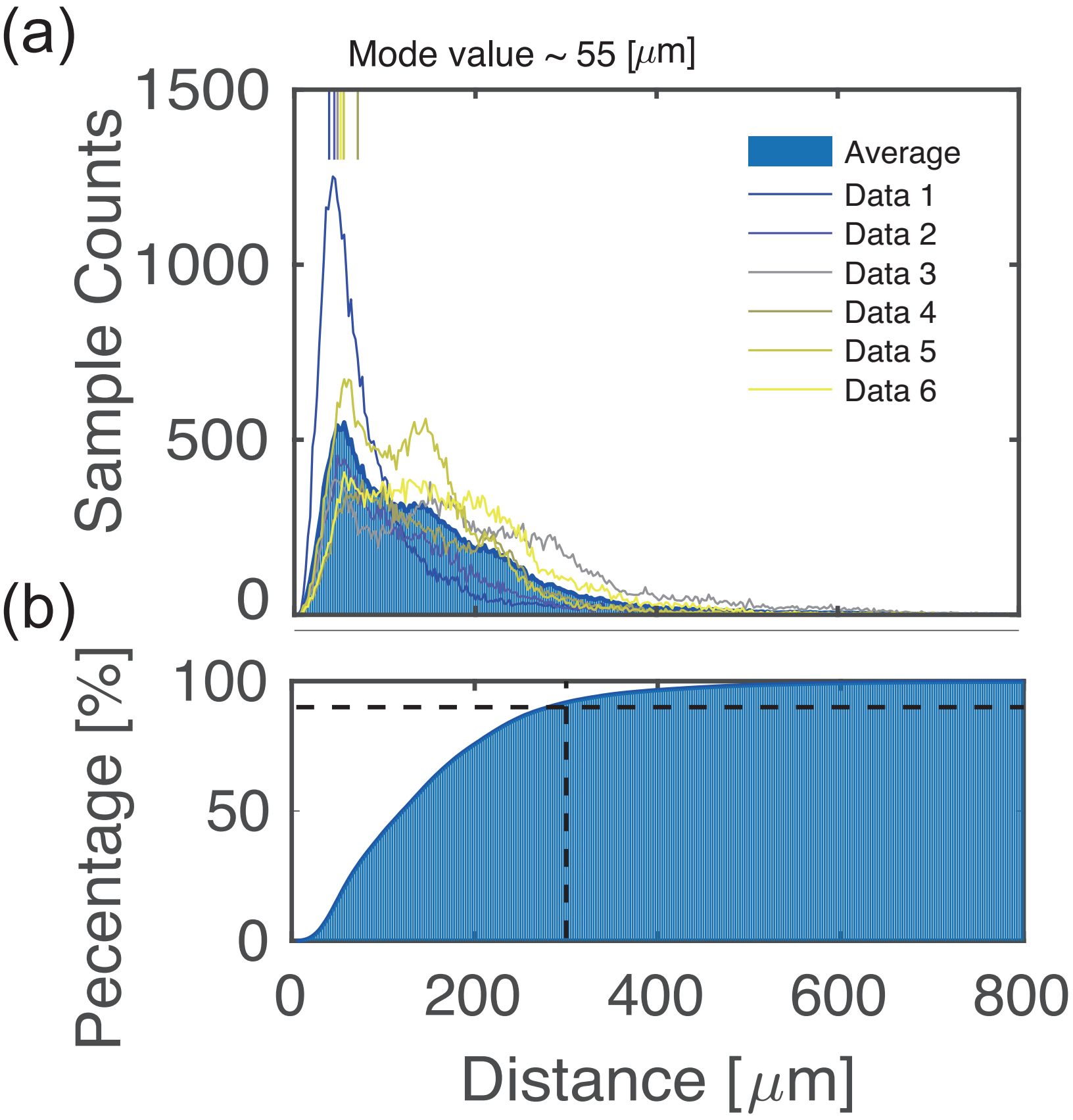


Figure4

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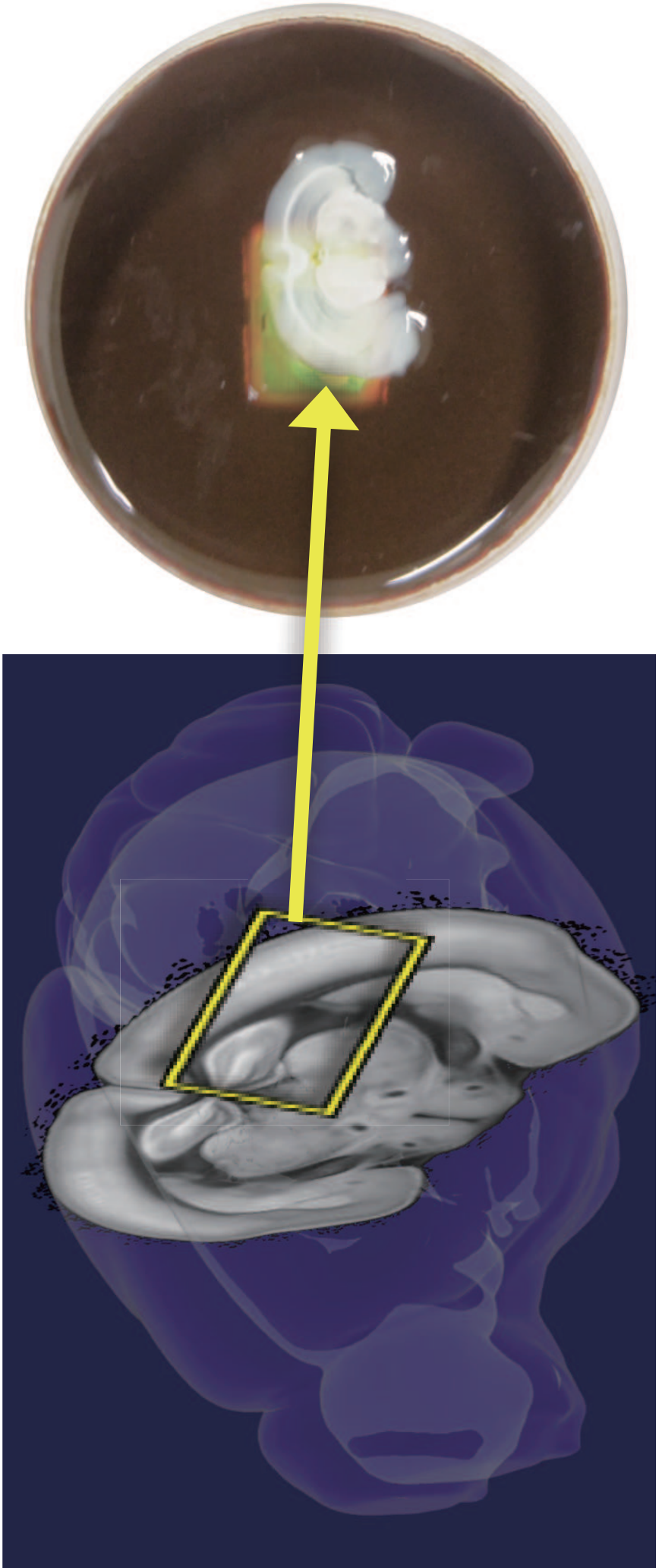

Table 1

Parameters	Values
Repetition time (TR)	2000 ms
Echo time (TE)	9 ms
Effective TE:	45 ms
RARE factor	16
Acquisition matrix size	196 x 144 x 144
Field of view (FOV)	19.6 x 14.4 x 14.4 mm^3
Acquisition bandwidth	75 kHz
orientation	Axial (coronal orientation in scanner setting)
Fat suppression parammeters	
fat frequency	2.6 ms-gaussian-shaped π/2 pulse with 1051 Hz bandwidth
spoiler gradient	
dummy scans	2 times
Number of averages	3
Acquisition time	2 h 42 min

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Air compressor	Kimura	KA-100	Animal preparation for MRI
All-in-one fluorescence	KEYENCE	BZ-X710	
Anesthesia box	Bio	RIC-01	Animal preparation for MRI
Anesthesia system	ACOMA	NS-5000A	Animal preparation for MRI
Anti-GAD67, clone 1G10.2	Merk	MAB5406	For immunostaining
Calcium Chrolide	nacalai	06729-55	aCSF
Choline Chloride	nacalai	08809-45	aCSF
curved blunt forceps			
Disposal scalpel	Kai	10	
D-PBS(-) without Ca and Mg, liquid(	nacalai		For immunostaining
D(+)-Glucose	Wako	049-31165	aCSF
Gelatin	nacalai	16605-42	re-secctioning
Goat anti-Mouse IgG (H+L) Highly	Invitrogen	A32723	For immunostaining
Goat anti-Rabbit IgG (H+L) Highly	Invitrogen	A32732	For immunostaining
Heater mat	Bio	HM-10	Animal preparation for MRI
Heater mat controller	Bio	BWT-100A	Animal preparation for MRI
Heater system	SA	MR-compatible	Animal preparation for MRI
Isoflurane	AbbVie		Animal preparation for MRI
Isoflurane vaporizer	ACOMA	MKIIIai	Animal preparation for MRI
Linear Slicer	DOSAKA	Neo Linear Slicer	
L(+)-Ascorbic Acid Sodium Salt	Wako	196-01252	aCSF
Magnesium Chrolide Hexahydrate	Wako	135-00165	aCSF
MaxOne Single-Well MEA	MaxWell		
Metal Spatula			
Monitoring system	SA	Model 1025	Animal preparation for MRI
Monitoring software	SA	PC-SAM V.5.12	Animal preparation for MRI
MRI compatible cradle	Bruker	T12812	Animal preparation for MRI
MRI coil	Bruker	T9988	For MRI
MRI operation software	Bruker	ParaVision 5.1	For MRI
Neo LinearSlicer MT	D.S.K.	NLS-MT	
NeuN (D4G40) XP Rabbit mAb	Cell	24307	For immunostaining

Normal Goat Serum	Wako	143-06561		For immunostaining
Potassium Chloride	Wako	163-03545		aCSF
Polyethylene Glycol Mono-p-isooct	nacalai	12967-45		For immunostaining
Pressure-sensitive respiration	SA	RS-301		Animal preparation for MRI
Preclinical MRI scanner	Bruker	BioSpec 70/20		For MRI
Pyruvic Acid Sodium Salt	nacalai	29806-54		aCSF
SCAN in a BOX	Open			
scissors				
Sieve bottle	TIGERCRO		81	For 3D scan
SlowFade Gold Antifade Mountant	Invitrogen	S36937		For immunostaining
Sodium Chloride	Wako	191-01665		aCSF
Sodium Dihydrogenphosphate	Wako	197-09705		aCSF
Sodium Hydrogen Carbonate	Wako	191-01305		aCSF
Sodium Hydrogensulfite	nacalai	31220-15		For immunostaining
Thermistor temperature probe	SA	RTP-101-B, PLTPC-		Animal preparation for MRI
Tooth bar	Bruker	T10146		Animal preparation for MRI
Winged intravenous needle	TERUMO	SV-23CLK		For perfusion
1 mol/l-Tris-HCl Buffer Solution	nacalai	35436-01		For immunostaining
1 mol/l-Hydrochloric Acid	nacalai	37314-15		For pH adjustment of solution
16%-Paraformaldehyde Aqueous So	Electron Mi		15710	For immunostaining

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Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. The manuscript should be professionally edited to be clear and free from grammatic mistakes.

We carefully checked English with a professional native speaker.

We hope the current version is nicely refined.

2. The title is unclear. Please revise.

The title was also changed significantly to make the main topic become clearer.

3. Please remove commercial language (DOSAKA Neo Linear Slicer MT, Triton X-100) and replace them with generic terms.

Thank you for point out the remained issues.

We removed those commercial languages.

4. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible.

We carefully corrected this point at whole of the protocol session.

Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol.

This point seems to be fine now.

Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However,

notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We eliminated “Notes” as well as possible. If you thought we can erase more at any place, let us know again.

Please move the discussion about the protocol to the Discussion.

I thought you are mentioning about the last sentence of the introduction section here;

Therefore, this indicates a high degree of accuracy in superimposing through correctly positioning micro data in the macro map. Finally, this proves that this experimental protocol will accurately position functional or structural micro-connectome, comprehensive micro-circuit architectures of individual neurons, correctly in the context of the whole-brain

Because this part is redundant even if we move it to the discussion section, we decided to simply cut this last sentence.

If you mentioned about the different part here, please ask again.

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Thank you for listing concrete examples.

We revisited all following points you suggested here.

6. 5.4: What are the recording materials?

This was just a dice, or something. Because this step is not essential,

We solved this problem with your question 8 together.

7. 6.2, 6.8: Please specify all surgical tools used.

Thank you for suggesting it. – We could clearly realize that we needed to write basic several tools here after hearing the comment.

8. 6.6: Please specify the previously selected parameters.

We moved the parameter information to here from the previous test recording step (step 5.4).

9. 6.11: Please specify the cutting speed and amplitude used in this step.

Thanks for asking it. We wrote the cutting speed and amplitude more clearly.

10. 6.14: What is the flow rate of the solution?

We added the information now.

11. 7.1.1: Please mention the recommended thickness of slice.

That is less than 50 micro meter. We added the information now.

12. Figure 1: Please describe panel f in the figure legend.

We added legend for figure 1-f now. Thanks for telling the failure.

13. Table 1: Please apply superscript to the number 3 in the unit (mm3). Please change the time unit in 42 m to “min”.

Thank you for suggesting them.　We corrected these points.

14. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

We prepared the sorted material list.

Reviewers' comments:

Reviewer #2: The authors addressed the major concerns/comments of the first review in an appropriate way. The manuscript is now written in readable English which improves understanding the protocol. Thanks for taking action in this respect.

Minor Concerns:

line 81: "There are, however, few systematic and reliable ways to make brain slices in an organized way." Please, give some examples/references here (e.g., Amunts, K., Lepage, C., Borgeat, L., Mohlberg, H., Dickscheid, T., Rousseau, M.E., Bludau, S., Bazin, P.L., Lewis, L.B., Oros-Peusquens, A.M., et al. (2013). BigBrain: An Ultrahigh-Resolution 3D Human Brain Model. Science 340, 1472 - 1475.; Ali, S., Worz, S., Amunts, K., Eils, R., Axer, M., and Rohr, K. (2018). Rigid and non-rigid registration of polarized light imaging data for 3D reconstruction of the temporal lobe of the human brain at micrometer resolution. Neuroimage 181, 235-251).

Thank you for suggesting these important jobs. We cited them now.

lines 511-513: Brain folding will be a strong limitation for the surface scans, since a large fraction of surface is hidden. In addition, the larger the brain tissue the

more likely are non-linear deformations. This will make registration much more challenging (non-linear registration required)!

Thank you for pointing out this issue. But, we should say that this is not true. Even though our brain has many sulci, we can overlap two brain surfaces using recording points on gyri. Therefore, this is practically not a serious issue.

Reviewer #5: Manuscript Summary:

The authors present a 3D scanning protocol to bridge macroscale brain anatomy

and microscale neuronal connections. The presented protocol is well detailed and is shown to achieve good results.

Major Concerns:

None

Minor Concerns:

1. Figure 3c is an important result figure Please label its necessary parts to convey the results clearly. If possible, add another result example in addition to 3c, may be an image/scan from a different angle of the brain. Also, re-write Figure 3c caption to correct grammatical errors and convey a clear summary of results achieved.

I'm sorry for causing the confusion.

As mentioned in the original caption, the old figure 3-(c) is a symbolic illustration, it

means the figure is not a result but a concept figure.

We are still developing a GUI for visualizing the embedded slices in the 3D space, we clearly mentioned it is an on-going project in the discussion section, and also separated the figure 3-(c) as figure 4 not to cause any more confusion.

However, we think the figure 3-a,b solidly proved that we can embed 300 μ m thick slices accurately into MRI space.

We hope to show you the GUI as soon as possible in the near future even though it is not now.

Thank you for helping to improve this report.

2. Add a colored cell legend at the bottom of Figure 2e.

Although I'm not sure what a colored cell legend mean, we wrote what color (red or green) corresponds with the two staining chemicals into the legend.

3. This work often refers to MEAs and their contribution to deciphering brain networks. This calls for citing implantable MEAs work too which has enhanced our understanding of brain networks in recent years. This should be mentioned in the introduction with some of the recent work like:

- Kozai et al, "Ultrasmall implantable composite microelectrodes with bioactive surfaces for chronic neural interfaces", Nature Mat. 2012.

- Kampasi et al, "Dual color optogenetic control of neural populations using low-noise, multishank optoelectrodes", Microsystems and Nanoengineering-Nature, 2018.

Thank you for suggesting these important jobs. We cited them now.

4. Word 'superbly' used twice on page 11, line 3.

I’m sorry for the basic mistake. We corrected it now.

5. Check grammar of the first three lines of 'Discussion' and the whole 'Discussion' section in general.

Yes. We checked English with a native speaker.

We hope you like the current version.