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3D reconstruction of brain section images for creating axonal projection maps in marmosets

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HIGHLIGHTS

- Axonal projections were reconstructed in 3D from brain section images.
- Brain section images were registered to their block-face images during sectioning.
- Registration error was estimated to be <200 μm.
- An ICA-based autofluorescence reduction method was proposed and found to significantly improve separation of fluorescent tracer signals from the background.
- The method can be combined with brain area annotation based on standard histology.

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ABSTRACT

Background: The brain of the common marmoset (Callithrix jacchus) is becoming a popular non-human primate model in neuroscience research. Because its brain fiber connectivity is still poorly understood, it is necessary to collect and present connection and trajectory data using tracers to establish a marmoset brain connectivity database.
New method: To visualize projections and trajectories of axons, brain section images were reconstructed in 3D by registering them to the corresponding block-face brain images taken during brain sectioning. During preprocessing, autofluorescence of the tissue was reduced by applying independent component analysis to a set of fluorescent images taken using different filters.
Results: The method was applied to a marmoset dataset after a tracer had been injected into an auditory belt area to fluorescently label axonal projections. Cortical and subcortical connections were clearly reconstructed in 3D. The registration error was estimated to be smaller than 200 μm. Evaluation tests on ICA-based autofluorescence reduction showed a significant improvement in signal and background separation.
Comparison with existing methods: Regarding the 3D reconstruction error, the present study shows an accuracy comparable to previous studies using MRI and block-face images. Compared to serial section two-photon tomography, an advantage of the proposed method is that it can be combined with standard histological techniques. The images of differently processed brain sections can be integrated into the original ex vivo brain shape.
Conclusions: The proposed method allows creating 3D axonal projection maps overlaid with brain area annotations based on the histological staining results of the same animal.

1. Introduction

The common marmoset (*Callithrix jacchus*) has been used as an animal model in neuroscience research, especially in studies of auditory processing (Eliades and Wang, 2008; Nelken et al.,

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2014; Miller et al., 2016) and affective processing (Dias et al., 1996; Roberts and Wallis, 2000; Roberts, 2011). Now, it is becoming a popular non-human primate model in neuroscience research (Okano et al., 2015; Miller et al., 2016; Izpisua Belmonte et al., 2015), including research areas such as vision (Mitchell et al., 2014; Hung et al., 2015; Suzuki et al., 2015a, 2015b), the motor cortex (Bakola et al., 2015), cortical development (Oga et al., 2013; Sasaki et al., 2015), and disease models (Kishi et al., 2014; Yasue et al., 2015), partially due to its compact brain size, well-developed prefrontal cortex, rapid maturation, and the availability of transgenic technologies (Sasaki et al., 2009). An investigation into in vivo cortical circuits has been started using two-photon microscopy with genetically engineered viruses (Sadakane et al., 2015a, 2015b). In order to study the underlying neural mechanisms in more detail, their anatomical connections must be revealed. For the marmoset, many retrograde neuroanatomical tracing studies are available (Rosa and Tweedale, 2005; Solomon and Rosa, 2014), while there is a general lack of anterograde tracing data.

In addition, earlier neuroanatomical tracing studies show results only from parts of brain sections, due to limited space in books or journals. A whole-brain database would greatly benefit from a 3D presentation of the data. This would improve understanding of trajectories and connection patterns in the whole brain (Majka et al., 2012), with high-resolution microscopic images of 2D brain sections for understanding precise axon morphology, trajectories, and termination patterns in cortical layers. In line with this, The Allen Mouse Brain Connectivity Atlas (http://connectivity.brain-map.org/) has been successful in showing brain connections in a comprehensive way, using a sophisticated user interface (Oh et al., 2014; Kuan et al., 2015). For this mouse brain atlas, the connectivity pattern labeled by fluorescent proteins was scanned using a TissueCyte 1000 serial two-photon tomography system (Ragan et al., 2012), which produced images of fluorescently labeled connections within a 100 μm depth volume every time before the brain was sliced using a microtome. This system eliminates concerns about tissue distortions in conventional histology and provides inherently pre-aligned images for precise 3D mapping. The obtained fluorescent images were registered to a standard mouse brain atlas for area annotations.

However, there are considerable individual differences in primate brain morphology. For example, in marmosets, the spatial relationship of the subdivisions in the prefrontal areas is consistent between individual animals, but the size and shape of the areas are variable (Burman and Rosa, 2009). Therefore, for the marmoset brain, the connectivity data collection strategy used in the mouse needs to be complemented by a histological analysis. One series of interleaving brain sections is used for connection analysis, and another series for area annotation, based on standard histological methods such as myelin and Nissl substance staining (Rosa et al., 2009).

The present study aims at creating a 3D reconstruction to show axonal projections with brain area annotations based on the histology of the same brain. In combination with histology, one way for achieving 3D mapping is the use of block-face images taken during sectioning (Annese et al., 2006; Dauguet et al., 2007a,b; Mailly et al., 2010; Choe et al., 2011). Using block-face images, several previous studies successfully created 3D reconstructions. However, to date, only one study examined the size of the reconstruction error (Choe et al., 2011). This information is relevant, because if the size of the error is comparable to or larger than the size of the brain areas (i.e., at the mm level in the marmoset), brain area annotations are inaccurate and meaningless. Therefore, we evaluated the error in 3D reconstructions created from block-face images. Another key difference between earlier studies and our work is the use of Advanced Normalization Tools (ANTs; Avants et al., 2011), which is a software package for image registration, implementing one of the best non-linear registration algorithms in terms of accuracy (Klein et al., 2009). We think that the accuracy of non-linear registration is important, because tissue shrinkage occurs in histological brain sections. Therefore, we chose the state-of-the-art algorithm for image registration.

Another issue to deal with is background autofluorescence of the tissue. Signals from fluorescently labeled neural processes and background autofluorescence of the tissue overlap and should be properly separated. For example, in the Allen Mouse Brain Connectivity atlas, morphological features or "edges" of neural processes were analyzed and detected as tracer signals. Separation must be achieved for high-resolution images at the μm level to reliably distinguish neural processes from each other. Processing many high-resolution images from a whole brain would be computationally demanding. Here we tested an easier approach using independent component analysis for images taken with different fluorescent filters to reduce autofluorescence that is present in all channels.

2. Materials and methods

2.1. Animals, tracer injection, and histology

All experimental procedures were approved by the Experimental Animal Committee of RIKEN or by the Experimental Animal Committee of the National Institute of Neuroscience and Psychiatry, and animals were handled in accordance with the "Guiding Principles of the Care and Use of Animals in the Field of Physiological Science" formulated by the Japanese Physiological Society.

The proposed method was applied to an actual tracer experiment result, where a viral tracer injected into the brains of three adult common marmosets (*C. jacchus*) had produced fluorescent-labeled axonal projections. During the surgery, anesthesia was administered by a ketamine hydrochloride injection (Ketalar, 25 mg/kg, i.m.), following an atropine injection (0.15 μg/kg, i.m.). Then the animal was intubated and artificially ventilated using a respirator (SN-487-3, Shinano Seisakujo, Tokyo, Japan) with sevoflurane or isoflurane (1.0–2.0%), N_2O (~70%) and O_2 (~30%). During surgery, vital signs such as rectal temperature, the electrocardiogram, and expired CO_2 were monitored. The head was fixed using a stereotaxic apparatus (SR-6C, Narishige, Tokyo, Japan). After craniotomy (~1 cm in diameter), a small incision was made in the dura. Using a manipulator (MM-3, Narishige, Tokyo, Japan), a glass pipette (outer diameter: 20–30 μm) attached to an injector (Nanoject II, Drummond Scientific Company, PA) was lowered and its tip was placed at a depth of ~800 μm below the cortical surface. After a 2 min waiting time, 300 nl of a viral tracer was injected at a rate of 25 nl/min. The viral tracer was a mixture of AAV1-Thy1S-tTA (titer: 1×10^9 vector genomes (vg/μl)) and AAV1-TRE-hrGFP (5×10^9 vg/μl), which expresses green fluorescent proteins in double infected cells and works as an anterograde fluorescent tracer, as confirmed in marmosets (Sadakane et al., 2015b). The pipette was withdrawn from the injection site after 5 min. The bone was put back and sealed by a thin layer of dental cement (Repeasin, GC, Tokyo, Japan). The skin was sutured. An anti-inflammatory drug (predonisolone, 1 mg, i.m.) and an antibiotic (cefovecin, 8 mg/kg, i.m.) were administered. After the animal regained consciousness, and following a thorough examination, it was returned to its home cage. An analgesic (meloxicam, 0.075 mg) was administered after the surgery (i.m.) and once a day for the following 3 days (p.o.).

The present study adopted the stereotaxic coordinate system used in a marmoset atlas (Paxinos et al., 2012); the horizontal zero plane, the anterior-posterior zero plane, and the left-right zero plane were defined as the plane passing through the lower margin of the orbit and the center of the external auditory meatus, the