Simultaneous acquisition of neuronal morphology and cytoarchitecture in the same Golgi-stained brain

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Abstract: Acquiring an accurate orientation reference is a prerequisite for precisely analysing the morphological features of Golgi-stained neurons in the whole brain. However, the same reflective imaging contrast of Golgi staining for morphology and Nissl staining for cytoarchitecture leads to the failure of distinguishing soma morphology and simultaneously co-locate cytoarchitecture. Here, we developed the dual-mode micro-optical sectioning tomography (dMOST) method to simultaneously image the reflective and fluorescent signals in three dimensions. We evaluated the feasibility of real-time fluorescent counterstaining on Golgi-stained brain tissue. With our system, we acquired whole-brain data sets of physiological and pathological Golgi-stained mouse model brains with fluorescence-labelled anatomical annotation at single-neuron resolution. We also obtained the neuronal morphology of macaque monkey brain tissue using this method. The results show that real-time acquisition of the co-located cytoarchitecture reference in the same brain greatly facilitates the precise morphological analysis of Golgi-stained neurons.

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OCIS codes: (110.0110) Imaging systems; (110.6880) Three-dimensional image acquisition; (120.5700) Reflection; (180.2520) Fluorescence microscopy.

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1. Introduction

One hundred years ago, Ramón y Cajal improved and used the Golgi staining method, named after its inventor [1], to show that spines [2] differentiate several neural categories [3] and defined a revolutionary paradigm stratagem to study neuron morphology. While various methods provide access to probe neuronal structures [4], many neuroscientists still utilize the classical approach of Golgi staining and its variations to visualize neural morphology under light microscopy. The staining efficiency of less than 5% [5, 6] makes it easy to distinguish classical approach of Golgi staining and its variations to visualize neural morphology under light microscopy. The staining efficiency of less than 5% [5, 6] makes it easy to distinguish the relatively sparse Golgi-stained neurons from intricate neural networks. The Golgi staining is used frequently for morphological studies, especially in primates, because of the lack of other efficient labelling technologies.

Anatomic features of neurons differ depending on their location in various brain regions. Acquiring an accurate anatomical reference is extremely important for precise morphological analysis of Golgi-stained structural information. Cytoarchitecture is commonly used as an anatomic reference and is considered the gold standard for anatomical annotation. Nissl staining has previously been employed to visualize cytoarchitecture for Golgi-stained brain slices. This method allows the acquisition of neural histological locations but loses the neuronal morphology because of deimpregnation of Golgi silver before counterstaining [25–27]. Nadia Pilati et al. [28] modified the Nissl staining method and overcame this obstacle. However, these protocols provide reflective contrast for imaging, as do Golgi staining. The modified method also fails to distinguish soma morphology or simultaneously co-locate

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cytoarchitecture in the same reflective channel. Therefore, acquiring an anatomical reference for Golgi-stained brain tissue requires counterstaining cytoarchitecture by fluorescent labels in the same brain. An ideal solution is to acquire a fluorescence-labelled anatomic reference simultaneously during whole-brain single-neuron imaging, as has been obtained in C. elegans and Drosophila brains [29, 30]. Uniform counterstaining in the Golgi-stained whole mammalian brain is still challenging. Extremely slow penetration of the counterstaining dye in Golgi-stained tissue results in additional counterstaining time of at least ten days to stain the whole mammalian brain, which reduces potential applications. No current technique can provide a fluorescence-labelled anatomic reference for locating single Golgi-stained neurons and image them simultaneously in the same brain.

Our group previously developed a real-time counterstaining method [31] to stain cytoarchitecture during imaging with propidium iodide (PI) to simultaneously acquire an anatomical reference for fluorescence-labelled neurons in the same brain. If we introduce this strategy to provide orientation information for Golgi-stained morphology, we must address two problems. The imaging contrasts of Golgi- and PI-stained signals were reflective and fluorescent, respectively. No current technique is capable of simultaneously obtaining reflective and fluorescent images. Moreover, the penetration law of PI molecules in Golgi-stained samples is still unknown; therefore, it is necessary to determine the feasibility of staining Golgi-stained brain tissue with PI in real time.

In this study, we propose a dual-mode Micro-Optical Sectioning Tomography (dMOST) method, which combines real-time counterstaining to image both the Golgi-stained morphology of neurons and fluorescence-labelled cytoarchitecture in the same brain at single-cell resolution. We employed a triple-window filter to transmit and split the illuminating, reflective and fluorescent beams. We studied the penetration speed of the PI solution in Golgi-stained brain tissue and optimized the acquisition strategy for obtaining whole-brain data. Using this method, we acquired 3D high-resolution data sets of Alzheimer’s disease (AD) model and control mouse brains and compared the dendritic morphological features of the Golgi-stained neurons with co-located landmarks in the cortex. We also imaged the prefrontal cortex of a macaque brain, demonstrating the potential for use in large-sized primate brain tissue. The dMOST method can facilitate Golgi-stained morphology study in neuroscience applications. Combined with other modified fluorescence labelling technologies, such as immunohistochemical staining, this method may provide cell type-specific molecular information for Golgi-stained morphological analysis.

2. Materials and methods

2.1 Animals

The animal experiments were approved by the Institutional Animal Ethics Committee of Huazhong University of Science and Technology, and all experiments were performed in accordance with relevant guidelines and regulations. A 2-month-old C57BL/6J male mouse, a 12-month-old C57BL/6J male mouse (Jackson Laboratory, 000664, Bar Harbor, ME, USA), a 12-month-old APP/PS1 mouse [32] (Jackson Laboratory, 005864, Bar Harbor, ME, USA), and an 8-year-old female macaque (Kunming Institute of Zoology, Chinese Academy of Sciences, 07388, China) were used.

2.2 Tissue preparation

All histological procedures have been previously described [33]. Briefly, the mice were anesthetized with ethyl carbamate and chloral hydrate, and the entire brain was removed and then immersed in fresh Golgi-Cox solution [34] for fixation and staining. The subsequent sample preparation followed the same protocol. The Cox solution was replaced the next day and then monthly to complete 2 months of impregnation. After staining, the brain was immersed in 1% lithium hydroxide (LiOH) for 1 d and then rinsed with double distilled water.
for 1 d. The rinsed brain was dehydrated for 2 h each with 50%, 70%, 85%, 95% and 100% ethyl alcohol in sequence. After dehydration, the brain was permeated in 50% and 70% LR-White resin (Ted Pella, USA, dissolved in absolute ethyl alcohol), each for 8 h, followed by 100% LR-White resin for 1 d. The brain was then totally immersed in a gelatine-sealed mould filled with 100% LR-White resin. After 3 d, the mould was put into a 55 °C oven for 36 h to induce polymerization.

The macaque brain tissue blocks were acquired from Prof. Hu Xintian’s lab in Kunming Institute of Zoology, Chinese Academy of Sciences, China. The subsequent sample preparation followed the same protocol with different parameters. The brain block was immersed in fresh Golgi-Cox solution for fixation and staining. The Cox solution was replaced the next day and then monthly to complete 3 months of impregnation. After staining, the brain block was immersed in 1% LiOH for 2 d and then rinsed with double distilled water for 2 d. The rinsed brain was dehydrated for 12 h each with 50%, 70%, 85%, 95% and 100% ethyl alcohol in sequence. After dehydration, the brain block was permeated in 50%, 70% and 100% LR-White resin (Ted Pella, USA, dissolved in absolute ethyl alcohol), each for 1 d, followed by 100% LR-White resin for 5 d. The brain block was then totally immersed in an airtight mould filled with 100% LR-White resin. Then, the mould was put into a 55 °C oven for 36 h to induce polymerization.

2.3 dMOST system

To simultaneously acquire Golgi- and PI-stained distribution information, we proposed a dMOST system to achieve automated high-throughput reflective and fluorescent imaging of large tissue at the same time, as shown in Fig. 1(a). In general, a beam splitter and dichroic mirror are employed in epi-illumination microscopes to split illumination and the signal beam for reflective and fluorescent imaging, respectively. For dual-mode imaging, researchers must switch the dichroic mirror and beam splitter to alternatively acquire reflective and fluorescent imaging. Here, we proposed a triple-window filter as the beam splitting component for dual-mode imaging. In general, dichroic mirror contains a reflecting window for the excitation beam (the first window) and a transmitting window for the fluorescent signal (the second window). In contrast, this filter consisted of a transflective window (the first window) for illumination and reflection signals of Golgi-stained neurons, a reflecting window for the fluorescence excitation beam (the second window), and a transmitting window for the fluorescent emission signal of the PI-stained signal (the third window). We chose an off-the-shelf dichroic mirror (DM1, FF560-Di01, Semrock Inc., Rochester, New York, America) as the beam splitting component in this system. The first window reflected and transmitted the light from 400 to 450 nm to illuminate and image the Golgi-stained signals. Matching an excitation filter (Ex, BSP01-532R, Semrock Inc., Rochester, New York, American), the second window reflected the beam from 450 to 530 nm to excite PI molecules. The third window passed the fluorescence emission signal of the PI-counterstained tissue at the wavelength longer than 550 nm. Another dichroic mirror (DM2, FF562-Di03, Semrock Inc., Rochester, New York, America) and an emission filter (Em, FF01-665/150, Semrock Inc., Rochester, New York, America) were used to split and filter the reflective and fluorescent signals in the detection path, respectively. The rest of the imaging and sectioning parts were based on a previous prototype [31]. Samples were immersed in PI solution and fixed on a high-precision 3D translation stage (x/y/z scanning stage, Aerotech Inc., Pittsburgh, PA, USA) during data acquisition. Volumetric imaging was performed by a combination of structured illumination microscopy for optical-sectioning imaging and objective movement for z scanning. After mosaic scanning in the x-y plane to cover the entire imaging range, the system moved the sample to the sectioning part and removed the imaged sample tissue. Next, a superficial layer of the sample was immediately exposed and penetrated by PI molecules from the solution. Only superficial tissue was stained and deep tissue was uncolored during the time interval between sectioning and imaging, avoiding background interference from
deep tissues in the fluorescence channel. The sample was then moved back to the imaging part, so the system continuously acquired the data of the next layer. Sectioning, staining and imaging were repeated in cycles until the whole sample had been imaged. We compared the real-time effects of PI counterstaining at the concentrations of PI solution of 1, 2 and 5 μg/ml (Life Science Technologies Corp., Shanghai, China). The different immersion time of 30, 90, 150 and 210 s at a PI concentration of 5 μg/ml were also compared to optimize our experiments.

To validate the performance of the dMOST system, we imaged standard samples to measure the lateral and axial resolution of both channels, as shown in Fig. 1(b). A 1951 United State Air Force (USAF) resolution target (2” × 2” Positive, Edmund, Barrington, NJ, USA) was imaged to obtain a lateral resolution measurement of the reflective channel. The lateral resolution of the reflective channel, as measured by the step-edge test method [35], was 0.36 μm. For the fluorescent channel, we measured red fluorescent beads with a diameter of 200 nm. The full widths at half maximum (FWHM) of the intensity profile of the beads were calculated, and the lateral resolution of the fluorescent channel was 0.60 μm. The lateral resolution of the fluorescent channel was longer than the reflective channel due to the longer wavelength of the fluorescence signal. The axial responses (optical sectioning thickness) of both channels were determined by the modulation period of structured illumination [36, 37]. We used a pattern period of 54.72 μm (13.68 μm/pixel × 4 pixel, half on and half off) on the digital micromirror device (DMD) and shifted three times with a phase shift of π/2 to generate an image with an optical-sectioning thickness of 2.0 μm [36]. We detected the reflective intensities of a mirror along the z axis, scanning from −10 μm to + 10 μm in 0.1 μm steps (0 μm represented the focal plane of the system). The FWHM of the reflective axial response was 2.2 μm (mirror). Then, we acquired the axial intensity profiles of the fluorescent beads and calculated the axial response of the fluorescence channel, which was 2.58 μm, which may result from the mismatching of the refractive index of the coverslip and the slightly low performance of the high NA objectives [38, 39].

![Fig. 1. dMOST system. (a) Configuration. CL, collimating lens; DMD, digital micromirror device; TL1, TL2, tube lens; Ex, excitation filter; Em, emission filter; DM1, three-working window dichroic mirror, DM2, dichroic mirror; PZT, piezoelectric translational stage. (b) Lateral and axial resolutions for both channels. I_F: fluorescence intensity; I_R: reflection intensity.](image)
3. Results

3.1 PI immersion principles

To evaluate the potential for real-time counterstaining of the Golgi-stained samples, we studied the counterstaining effects of PI in Golgi-stained brain tissue at different penetration concentrations and times, as shown in Fig. 2. The sample was immersed in PI solutions of 1, 2 and 5 μg/ml. We acquired the images of the same field of view (FOV) at the top surface after sectioning at 30, 90, 150 and 210 s. The light source was turned off during the imaging intervals to avoid unwanted bleaching due to prolonged exposure. For each concentration, the sample was sectioned at 20 μm to eliminate the residual influence of the previous staining. Figure 2(a) shows that the longer the penetration time, the more obvious the staining effect. Similarly, higher concentrations improved the staining effect and increased the signal to background ratio at the same immersion time. In particular, even at the shortest immersion time of 30 s, PI counterstaining at 5 μg/ml provided adequate imaging contrast for cytoarchitecture imaging (Fig. 2(b)). The results illustrated that a PI concentration of 5 μg/ml was preferred in our experiments.

Fig. 2. PI penetration of Golgi-stained brain tissue. (a) Images of the same FOV of the sample immersed in the PI solution at the specific concentrations (1, 2 and 5 μg/ml) for different immersion times (30, 90, 150 and 210 s). Scale bar, 50 μm. (b) Normalized intensity profiles of the same cells indicated by white lines in the same row in (a) at different immersion times for each concentration.

3.2 Imaging depth

Metal staining with the Golgi method makes the Golgi-stained neurons have stronger reflectivity than the surrounding brain tissue. The strong reflection of Golgi-stained signals allowed us to image a block of tissue with z scanning. Thus, increasing the imaging depth in a single FOV could save the time of repeat mosaic scanning at different depths, thereby shortening the total data acquisition time. However, light absorption and scattering in deep tissue might result in poor image quality. To determine the possible maximum imaging depth, we compared the images acquired by optical and mechanical sectioning at different depths (Fig. 3(a)). First, we obtained serial optical-sectioning images from a 50 μm-depth tissue block at a z scanning step of 1 μm. Then, we employed the circle of optical imaging and mechanical sectioning to acquire serial surface images of the same FOVs at the same depths with an interval of 1 μm (mechanical-sectioning images). Typical results for the optical- and mechanical-sectioning images and their merged images at the depths of 15, 20 and 25 μm are shown in Fig. 3(b). The overlap of the optical- and mechanical-sectioning images at the depth
of 15 μm demonstrated that there was no signal loss associated with imaging at this depth by optical scanning. However, at 20 μm, the optical-sectioning image partially lost signal compared with the mechanical-sectioning image. At the depth of 25 μm, this signal loss became more obvious. The results illustrated that an optical scanning depth of 15 μm was suitable in our system to guarantee image quality and signal integrity.

Fig. 3. Imaging depth. (a) Schematic diagram of the acquisition of optically and mechanically sectioned images. (b) Optically and mechanically sectioned images and their merged images at the depths of 15, 20 and 25 μm. White arrows indicate the signal differences at the same positions between the optically and mechanically sectioned images. Scale bar, 5 μm.

3.3 Surface flatness at different sectioning thicknesses

To validate the maximum thickness of mechanical sectioning by diamond knife, we measured the surface flatness of Golgi-stained resin-embedded brain tissue at sectioning thicknesses from 1 to 5 μm (Fig. 4). Sectioning speed and width were 300 mm/min and 2 mm, respectively. After each section was cut, we immediately measured the flatness of the sample surface along the sectioning direction with a stylus profiler (Dektak XT, Bruker, Karlsruhe, Germany). Sectioning resistance increased with the increased section thickness, inducing the deformation to the sample and leading deterioration in sectioning quality. The friction between the knife and the sample would be increased as the sectioning thickness increased, introducing the undesired longitudinal deformation to the tissue during the cutting process, which led to poor cutting quality. Thus, the surface roughness increased with the section thickness, as shown in Fig. 4(a). The root mean square (RMS) was 19.7 nm at a sectioning thickness of 1 μm, which is consistent with a previous study [33]. When the sectioning thickness increased to 2 and 3 μm, there were tiny fluctuations on the sample surface, and the RMS were 44.2 nm and 97.9 nm, respectively. Excellent slice quality still allowed us to acquire images at the top sample surface, as shown in Fig. 4(b) and 4(d). The RMS increased to 947.9 nm and 2.1 μm at sectioning thicknesses of 4 and 5 μm, respectively. This increased RMS might lead to possible signal losses when imaging at the top sample surface, as shown in Fig. 4(c) and 4(e).
3.4 Dual-channel signal acquisition

The strong reflectivity of Golgi-stained signals provided a possibility for imaging by axial scanning and the use of thick tissue sections, which would shorten data acquisition time. However, the limited penetration depth of PI at the superficial surface required smooth surface flatness at an ultrathin sectioning thickness for imaging the PI signals. To optimize data acquisition efficiency, we proposed two kinds of acquisition strategies to simultaneously acquire interval-sampling or full-volumetric PI-stained cytoarchitecture for Golgi-stained tissue. Standard brain atlas, such as the Allen brain atlas [40], consists of images of 100 μm-interval cytoarchitecture sections for identifying brain regions. We proposed to acquire the PI counterstaining signal at the sampling interval for only brain region segmentation (fast acquisition approach), while a full-volumetric acquisition was preferred for orienting neurons at a single-cell resolution (full acquisition approach). The fast acquisition approach had the advantage of saving data acquisition time. The information of the spaced cytoarchitectural images sufficiently enabled the identification of brain regions in spite of enabling to some detailed cellular analysis, such as calculating a ratio of Golgi-stained neurons to all cells. Whereas the full acquisition approach enabled the acquisition of the whole cytoarchitecture for the Golgi-stained signal.

To demonstrate the fast acquisition approach, we imaged a 12-month-old Golgi-stained C57 mouse brain at a voxel resolution of 0.32 × 0.32 × 1 μm with 50 μm interval-sampled cytoarchitecture information in 103 hours (Fig. 5). The exposure time of the Golgi-stained and PI-counterstained signals were 1 and 3 ms, respectively. The raw data set was 15
terabytes, including 13,380 coronal sections for the reflective channel and 267 coronal sections for the fluorescent channel. Figures 5(a)-5(c) show a Golgi-stained coronal plane in the Bregma −2.7 mm, its co-located PI-counterstained landmarks and their merged image, respectively. The cytoarchitecture allowed for the identification of the location of Golgi-stained signals (Fig. 5(b)). Figures 5(d)-5(f) show the raw data of the Golgi-stained and PI-counterstained signals and as their merged image of the enlarged view of the region indicated by white boxes in Fig. 5(a)-5(c), respectively. The overlapping reflective and fluorescent signals are indicated by yellow arrowheads in Fig. 5(d)-5(f), which illustrate our single-cell orientating precision at the lateral resolution. The results indicated that the simultaneous acquisition of cytoarchitecture made it possible to study Golgi-stained neuron morphology of different brain regions.

To demonstrate our ability to obtain a full-volumetric data set of neural morphology and co-located cytoarchitecture of a Golgi-stained mouse brain, we imaged brain tissue from a 12-month-old APP/PS1 transgenic mouse at the voxel resolution of 0.32 × 0.32 × 1 μm in 244 hours. The raw data set was 28 terabytes, including 13,265 coronal sections for each channel. We reconstructed the Golgi-stained and PI-counterstained images of the midsagittal plane (approximately lateral, 1.35 mm) of the brain, from the olfactory areas (OLF) to hindbrain (HB), as shown in Fig. 6(a). The main regions were identified by referring to the Allen brain atlas [40]. The reconstructed continuous fibres illustrated the data integrity and acquisition stability of our system. To our knowledge, this is the first study to show Golgi-stained neurons with their co-located cytoarchitecture in the resliced sagittal plane. The enlarged views of Fig. 6(b) and c show that the PI-counterstained signal contributed to the segmentation of brain regions for Golgi-stained signals. We reconstructed two Purkinje cells in the CB (Fig. 6(d)). The high resolution allowed us to identify dendritic spines in the dendrites of the Purkinje cells. The overlapping Golgi-stained somas and PI-counterstained

![Fig. 5. Imaging the Golgi-stained and PI-counterstained mouse brain. (a) A 50-μm maximum intensity projection of Golgi-stained coronal plane in the Bregma, −2.7 mm. (b) An image of the PI-stained signal of the same coronal plane with 1 μm-thickness. Regions in the left brain were distinguished by referring to the Allen brain atlas [40]. Abbreviations: HIP, Hippocampal region; MB, Midbrain; TH, Thalamus; HY, Hypothalamus; CTXsp, Cortical subplate; and OLF, Olfactory areas. (c) The merged image of (a) and (b). (d) - (f) Enlarged views of the raw signals indicated by the white boxes in (a) - (c), respectively. Scale bar: (a) - (c), 1 mm; (d) - (f), 50 μm.](image)
nuclei in single planes demonstrated the single-neuron location accuracy (Fig. 6(e)-6(f) and 6(g)-6(h)). PI-counterstained anatomical reference (Fig. 6(f) and 6(h)) indicated that the Purkinje cells were located between the molecular layer and granular layer of cerebellar lobules IV-V. The results show that our method provides the potential to study Golgi-stained neuron morphology with co-located cytoarchitecture.

3.5 Neural morphological analysis of pathological and physiological model mouse brains

The accurate localization reference enabled the analysis and study of the neuronal morphological differences in specific brain regions for pathological and physiological models. We further quantified the neuronal features in the dentate gyrus (DG) of the hippocampus of the APP/PS1 transgenic mouse brain and compared it with its wide-type control at the same age (Fig. 7). Golgi-stained signals of the two brains exhibited a distinct difference (Fig. 7(a) and 7(c)), though the selectivity of Golgi staining was random [41, 42]. Co-located PI-counterstained signals allowed us to compare the Golgi-stained neurons at similar coronal planes in the two brains (Fig. 7(b) and 7(d)). The enlarged views of the DG areas showed that neuron number and morphology of the AD mouse brain were obviously different from the control mouse brain (Fig. 7(e) – 7(h)). Further enlargement of the DG areas (Fig. 7(i) and 7(j)) showed the morphological changes of APP/PS1 mouse more clearly.
Granule neurons in Fig. 7(i) had less dendritic arborization than those in Fig. 7(j). We randomly reconstructed 10 granule neurons from the DG areas in each brain and quantitatively compared their dendritic morphology (Fig. 7(k)). We observed a loss in both total dendritic length and dendritic arborization in this AD model mouse brain, comparing with the control mouse brain. This result demonstrated that co-located PI-counterstained cytoarchitecture may provide a precise analysis of the neural features of pathological and physiological models with accurate orientation.

Fig. 7. Neuron morphology contrast of 12-month APP/PS1 and wild-type mouse brains. (a) and (b) Golgi-stained and PI-counterstained images in the left hippocampal coronal plane of the APP/PS1 transgenic mouse brain. (c) and (d) Golgi-stained and PI-counterstained images of a similar coronal plane of the controlled wild-type mouse. (e) - (h) Enlarged views of the regions in white boxes in (a) - (d), respectively. (i) and (j) Enlarged views generated by merging the images of regions in the white boxes in (c) and (f) as well as in (g) and (h), respectively. (k) Histograms of total dendritic length and dendritic arborization of the randomly selected granule neurons (n = 10) in (e, g). The significant differences between pairs are indicated by the p value (unpaired two-tail t-test, *** respresents p < 0.001). Scale bar: (a) - (d), 1 mm; (e) - (h), 200 μm; (i) and (j), 20 μm.

3.6 Imaging the prefrontal cortex of Golgi-stained macaque brain tissue

The study of primates is very important to our understanding of human brain function. Due to the lack of the effective fluorescent labelling for primate brains, Golgi staining is still an important staining method in the study of primate neuronal morphology. Therefore, we also imaged a block with dimensions of 27 × 22 × 2 mm from the prefrontal lobe of an 8-year-old macaque brain by full acquisition approach over 11 days to acquire the Golgi-stained neuronal morphology with cytoarchitecture of the prefrontal cortex in a primate brain (Fig. 8(a)-8(b)). The main regions were identified by referring to the Rhesus Monkey Brain atlas [43]. Enlarged views shown in Fig. 8(c) and 8(d) show that the PI-counterstained signal contributed to the segmentation of brain regions for the Golgi-stained signals. Figure 8(e) and 8(g) showed varying neuronal morphology of the different layers of areas 6DR (F7) and 6VR (F5), respectively. The PI-counterstained cytoarchitecture landmarks revealed that cortical neurons had laminar organization based on cell density differences [44, 45] (Fig. 8(f) and 8(h)). Figure 8(i) and 8(j) show the 3D view of a reconstructed neuron and its raw data location in layer II/III of Fig. 8(f), respectively. The enlarged view of the apical dendrites in the fuchsia box of Fig. 8(i) is shown in Fig. 8(k), and the spines are clearly distinguished. A quantitative comparison of the dendritic morphology, including total dendritic lengths, total dendritic branches, apical and basal dendritic branches, and apical spine densities, at layer
II/III of areas 6DR (F7) and 6VR (F5) is shown in Fig. 8(l). The results indicated that neurons in F7 were longer and more branched than those in F5, especially the apical dendrite branches. In contrast, the apical spine density in F5 was slightly denser than that of the F7 area. These results show the morphological diversity and regional difference of type-specific neurons in the primate cortex. Together, these results demonstrate that the high throughput and high resolution enabled our method to facilitate the study of neural morphology and cell type in primate brains. We also note that the size of the prefrontal tissue block in this proof of principle was larger than the size of the mouse brain. The maximum imaging range of the system was limited by the maximum travel range of the 3D translation stage. Imaging a half or whole primate brain at the same voxel resolution will inevitably lead to the extension of the data acquisition time and the increase of the data set size. It is a big challenge for not only imaging and sectioning but also labelling and data processing. It may need to further improve the imaging speed of our system.

Fig. 8. Localization and reconstruction of Golgi-stained neurons in the prefrontal cortex of the macaque brain. (a) Coronal plane of Golgi-stained neurons at the prefrontal cortex region of the macaque brain, approximately the Bregma 3.6 mm. The projection thickness was 50 μm. (b) Co-located PI-counterstained cytoarchitecture of (a). Regions were distinguished by referring to the Rhesus Monkey Brain atlas [43]. Abbreviations: 6M, area 6 of the cortex, medial part; 6DR (F7), area 6 of the cortex, dorsorostral part; 6VR (F5), area 6 of the cortex, ventral part, rostral subdivision; 8AD, area 8 of the cortex, anterodorsal part; 8AV, area 8 of the cortex, anteroventral part; 8B, area 8B of the cortex; 9/46, area 9/46 of the cortex; 44, area 44 of the cortex; 45B, area 45B of the cortex; Acb, nucleus accumbens; Cd, caudate nucleus; eg, cingulum; Cl, claustrum; cr, corona radiate; ec, external capsule; Gu, gustatory cortex; iar, inferior arcuate sulcus; ic, internal capsule; If, lateral fissure; OPro, orbital proisocortex; ProM, promotor area; ps, principal sulcus; Pu, putamen; sar, superior arcuate sulcus. (c) - (h) Enlarged views of signals indicated by the white boxes in (a) - (b), respectively. (i) 3D view of the reconstructed pyramidal neuron indicated by the blue arrows in (g). P, posterior; D, dorsal; M, middle. (j) A total of 300 × 700 × 50 μm raw data of yellow dash-line boxes areas in (i). Blue arrows indicate the reconstructed neuron of (i). (k) Enlarged view of apical dendrite spines indicated by the fuchsia box in (j). (l) Histograms of total dendritic length, mean branch length, total dendrite branches, apical dendrite branches, basal dendrite branches, and apical dendrite spines of randomly selected pyramidal neurons (n = 15) in layer II/III in (e) and (g) yellow box areas. The significant differences between pairs are indicated by the p value (unpaired two-tail t-test, ** respresents p < 0.01 and *** respresents p < 0.001). Scale bar, (a) - (b), 2 mm, (c) - (d), 500 μm, (e) - (h), 100 μm; (k), 10 μm.
4. Discussion and conclusion

Here, we report a whole-brain imaging method for precisely reconstructing and accurately orientating Golgi-stained neurons in 3D. We proposed that dMOST would achieve simultaneous acquisition of reflective Golgi-stained neurons and fluorescent PI-counterstained landmarks in the whole brain. We imaged Golgi-stained AD and control mouse brains and compared the morphological features of neurons in the hippocampus of these brains. In addition, we acquired a data set of brain tissue from the visual cortex in the primate brain and precisely reconstructed type-specific neurons on the co-located anatomical annotation. Our results demonstrate that our method was capable of accurately locating and reconstructing Golgi-stained neuronal morphology in pathological and physiological models of rodent and primate brains.

Our method provides co-located fluorescent anatomical reference for Golgi-stained brain tissues by real-time counterstaining. It overcame the inability of Golgi and Nissl staining to simultaneously visualize reflective morphology and cytoarchitecture. Due to the benefit from real-time counterstaining, we avoided additional time-consuming sample preparation. dMOST employed a triple-window filter for splitting each channel to achieve parallel acquisition of both reflective and fluorescent images, rather than alternate imaging by switching filter sets, which saves half the imaging time. This time reduction is extremely significant for whole-brain imaging. Even for the mouse brain, the whole-brain data set includes millions of FOVs, and the single-channel data acquisition lasted a couple of days. Furthermore, the strong reflection of Golgi-stained signals allowed us to image a block of tissue with z scanning. In this way, increasing section thickness and decreasing section numbers could save sectioning time, thereby shortening the total data acquisition time. Wide-field imaging also provided an advantage of high throughput, compared with line-scanning imaging used in a previous study [33]. The aforementioned advantages illustrate that our method holds potential for large-scale data acquisition for statistical morphological analysis based on the Golgi staining method.

Researchers have made important progress in the study of the rodent brain thanks to recent technical advances of innovative fluorescence labelling and optical imaging. However, primate brain labelling still relies on classical methods. Golgi staining is a crucial approach for studying neural morphology in primate brains. The visualization of Golgi-stained neurons with an anatomical reference by our method allows for extraction and comparison of morphological features of different targeted regions. In addition, high throughput and dual-channel imaging make our method more suitable for the large volumetric imaging of primate brains. We believe that our method will facilitate the study of the structural principles of brain function and disease in primates.

Funding

973 (project no. 2015CB755602), the NSFC (project no. 61421064, 91432105, 81671374 and 91232000), and the director fund of the WNLO.

Acknowledgements

We thank the members of the MOST group from the Britton Chance Centre for Biomedical Photonics for their assistance with experiments and comments on the manuscript. We are grateful to Ben Long for assistance with sample embedding and Wenjuan Shi, Xue Peng, Benyi Xiong, Yuxin Li, and Chongdi Guo for assistance with data processing. We appreciate Prof. Xintian Hu and Dr. Zhengbo Wang for providing the macaque brain tissue block for imaging. We also thank the Optical Bioimaging Core Facility of WNLO-HUST for support with data acquisition and the Analytical and Testing Centre of HUST for spectral measurements.
Disclosures

The authors declare that there are no conflicts of interest related to this article.