Neuroanatomical phenotyping of the mouse brain with three-dimensional autofluorescence imaging

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Gleave JA, Wong MD, Dazai J, Altaf M, Henkelman RM, Lerch JP, Nieman BJ. Neuroanatomical phenotyping of the mouse brain with three-dimensional autofluorescence imaging. Physiol Genomics 44: 778–785, 2012. First published June 19, 2012; doi:10.1152/physiolgenomics.00055.2012.—The structural organization of the brain is important for normal brain function and is critical to understand in order to evaluate changes that occur during disease processes. Three-dimensional (3D) imaging of the mouse brain is necessary to appreciate the spatial context of structures within the brain. In addition, the small scale of many brain structures necessitates resolution at the ~10 μm scale. 3D optical imaging techniques, such as optical projection tomography (OPT), have the ability to image intact large specimens (1 cm³) with ~5 μm resolution. In this work we assessed the potential of autofluorescence optical imaging methods, and specifically OPT, for phenotyping the mouse brain. We found that both specimen size and fixation methods affected the quality of the OPT image. Based on these findings we developed a specimen preparation method to improve the images. Using this method we assessed the potential of optical imaging for phenotyping. Phenotypic differences between wild-type male and female mice were quantified using computer-automated methods. We found that optical imaging of the endogenous autofluorescence in the mouse brain allows for 3D characterization of neuroanatomy and detailed analysis of brain phenotypes. This will be a powerful tool for understanding mouse models of disease and development and is a technology that fits easily within the workflow of biology and neuroscience labs.

Optical projection tomography; phenotype; whisker barrel; sexual dimorphism

THE STRUCTURAL ORGANIZATION of the brain is important for normal brain function, and evaluating its changes is critical in understanding disease progression. The brain and its components are inherently three-dimensional (3D); therefore, 3D visualizations of the brain are needed to appreciate the spatial context of the structures within it. This has led to the use of 3D imaging to monitor the changes that occur in the brain. Magnetic resonance imaging (MRI) and positron emission tomography combined with computed tomography are employed to image on a more macroscopic scale. Although these techniques are capable of imaging the entire brain, they often lack the image contrast or resolution necessary to visualize small structures, such as anatomic nuclei, or to localize signals to individual structures. Microscopic techniques, such as two-photon scanning laser microscopy and confocal microscopy, can achieve cellular and subcellular resolution (12, 24). However, these techniques lack the penetration depth to image the entire brain and are limited to imaging depths of only a few hundred microns. This is insufficient for imaging the complete mouse brain, the mammal most often used in biomedical research. Traditional histological sectioning is able to achieve cellular resolution over arbitrarily large volumes, but obtaining 3D data sets is time-consuming, and alignment of serial sections is notoriously difficult due to distortions that occur when slicing the tissue, making histological sectioning impractical for routine use.

For this reason, the development of 3D imaging methods that operate on a mesoscopic scale, with 5–50 μm resolution, over relatively large fields of view, approximately the size of a mouse brain, is of great interest. These imaging techniques can be used to study mouse brain organization at a critical scale between those offered by macroscopic and microscopic imaging. We have extensively explored the use of ex vivo MRI for this purpose (4, 5, 14, 15). Other alternatives include several 3D optical imaging techniques, which show promise at this scale and have the benefit of achieving cellular or near-cellular resolution with the ability to handle large specimens on the order of 10 mm³ or more. Such optical techniques include optical projection tomography (OPT) (21, 25), light sheet-based fluorescence microscopy (LSFM) (23), including single-plane illumination microscopy (25) and ultramicroscopy (UM) (9), high-resolution episcopic microscopy (HREM) (28), episcopic fluorescence image capture (EFIC) (25), and serial two-photon tomography (16) among others. In principle, these optical methods permit the use of standard immunofluorescent markers to visualize specific cell types in combination with tissue autofluorescence to visualize the neuroanatomy, which allows the distribution of cells to be determined within an anatomical context. A crucial component of these studies is the visualization, identification, and characterization of neuroanatomical differences between sample groups.

In this study we demonstrate the potential and the limitations of autofluorescence imaging at the mesoscopic scale for anatomical phenotyping of the mouse brain. We explored novel visualizations enabled by 3D autofluorescent imaging and tested the potential of computer-automated quantification for identification of subtle neuroanatomical differences. While we anticipate the results will be applicable to studies with other mesoscopic optical imaging methods, in this study we used OPT, which can resolve ~5 μm (27) in a 3D data set with ~1 cm² field of view within an imaging time of ~15 min. In OPT, optically cleared specimens are rotated 360°, and a series of projection images is collected. The projection images are then reconstructed into a final 3D image. The endogenous autofluorescence in the brain allows many structures to be visualized with optical imaging methods.

For our study, we first developed a protocol to achieve high-quality OPT images of the endogenous autofluorescence

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of the mouse brain, varying both the size of the samples and the concentration of fixative. Subsequently, we surveyed our brain images to determine the anatomical features that could be visualized. Finally, we used computer-automated methods to identify subtle neuroanatomical differences present between male and female wild-type mice.

METHODS

Specimen Preparation

**Animals.** All animal experiments were approved by the animal care committee of the Toronto Centre for Phenogenomics (Toronto, ON, Canada). Animals were maintained in standard housing conditions with five mice per cage (cages 7 in. width × 11 in. depth × 8.5 in. height in size). In preparation for perfusion fixation for all experiments, 8 wk old wild-type C57BL6/J mice (Toronto Centre for Phenogenomics, in-house breeding) were anesthetized with an intra-peritoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine.

**Specimen thickness studies.** To examine the effect of specimen thickness on OPT images, male mice were fixed with 4% paraformaldehyde (PFA) as described below. The hemispheres of the fixed specimens were divided and then sectioned using a coronal brain mold (Kent Scientific, Torrington, CT) or a vibratome (Leica). For this study, we used section thicknesses of 0.5 mm (n = 10), 1.0 mm (n = 10), 4.0 mm (n = 3), and 6.0 mm (n = 2).

**Fixation studies.** To study the effect of fixation on the image quality, male mice were perfused with 4% PFA (n = 2) or 1% PFA (n = 2). For 1% PFA fixation, 15 ml of 0.1 M PBS containing 10 U/ml heparin (Sigma, Oakville, ON, Canada) was intracardially perfused followed by 15 ml of 1% PFA. The brains were removed from the skull and soaked in 1% PFA for 2 h, washed, and stored in 0.1 M PBS until imaging. For 4% PFA fixation, mice were perfused with 30 ml of 0.1 M PBS containing 10 U/ml heparin (Sigma) followed by 30 ml 4% PFA. The brains were soaked in the skull overnight in 4% PFA, washed, and stored in 0.1 M PBS until imaging. All perfusions were performed with a Pharmacia minipump at a rate of ~1 ml/min. Specimens were sectioned 6 mm thick using the coronal brain mold, and the hemispheres were divided.

**Sexual dimorphism studies.** To investigate the potential for optical imaging to be used to evaluate subtle phenotypic differences, mice were perfused with 1% PFA (n = 9 male, n = 9 female) as described above and subsequently sectioned to 6 mm thick samples using the coronal brain mold.

**OPT preparation.** For all components of the study, specimens were embedded in 1% agarose (Fisher Scientific, Ottawa, ON, Canada) and then dehydrated stepwise to 100% ethanol over the course of 3 days. They were subsequently cleared in BABB (1:2 benzyl alcohol, benzyl benzoate) for 2–3 days.

**OPT Imaging.**

**Image acquisition.** All images were acquired using a custom-built OPT scanner. We took 1,200 projection views over 360° using a λ = 425 ± 15 nm excitation filter and λ = 473 nm long-pass emission filter and a 1,024 × 1,024 field of view. Projection images were captured on a cooled charge-coupled device camera (QImaging, Surrey, BC, Canada). The microscope zoom was held constant for comparisons of different specimen preparation protocols. Images were reconstructed using SkyScan software, NRECON (Kontich, Belgium).

**Visualization.** One of the key benefits of 3D imaging is the ability to visualize structures of complex shape in their native 3D configuration. To visualize the cortex in this fashion, we segmented the cortex and computed curved lines traversing it according to Laplace’s equation with methods described in detail elsewhere (13). These lines intersect the edges of the cortex perpendicularly. Selection of a 3D surface at increments throughout the cortex then allows different layers in the cortex to be visualized. From the outer surface, layer 4 was identified as lying 30–40% of the distance through the cortex. We used this method to highlight the features of cortical layer 4 by mapping the OPT intensities onto the surface defined in this fashion.

**Registration Analysis**

Anatomical differences between the male and female brains were detected through a process of automated computer registration. The registration procedure included linear and iterative nonlinear stages (1, 10). At completion, we obtained individual transforms for each sample that brought all brain structures into alignment in an unbiased average position. After transformation of all images into this space, a mean of all image signal intensities produced a population average for the study sample. We used the deformations that transformed images into the average space for volumetric analyses by computing the determinant of the Jacobian matrix, which represents the volume change at each voxel as a multiplicative scale factor. For voxel-wise analyses, the logarithm of this scale factor was compared statistically across all samples. We also computed volumes of segmented structures. For this purpose a segmented anatomical atlas of 36 structures was registered to the population average, and then voxel-wise volume changes were integrated over the regions defined by each segmented structure to obtain the volume measurements.

**Statistical Analysis**

In computational analyses of differences in the male and female brains, statistical tests for volume changes were performed after correction of volume based on a mouse-specific scale factor intended to account for biological and preparation-induced variability of sample size. To obtain the mouse-specific scale factor, all structure volumes for each mouse were normalized by the sex-specific structure
mean. Subsequently, the average of these normalized volumes was computed for each mouse and used as the mouse-specific scale factor to correct the volumes globally. The differences in the logarithm of the Jacobian determinants were evaluated voxel-wise with a Wilcoxon test, accounting for multiple comparisons using a 10% false discovery rate (7). Similarly, we statistically compared the corrected volumes of male and female mouse brain structures with a Wilcoxon test, accounting for multiple comparisons using a 5% false discovery rate.

RESULTS

Autofluorescent Imaging: Specimen Size and Fixation Protocol Considerations

We first assessed the contribution of brain specimen size and fixation protocol on our ability to image the mouse brain with optical methods, and specifically with OPT. To assess the influence of size on overall image quality, we used OPT to image sections of the mouse brain that varied in thickness (0.5–6 mm). We observed a greater amount of detail in 0.5 mm sections compared with thicker sections (Fig. 1). Histograms of the image signal intensities in cortical regions of interest (ROIs), for example, confirm that there is more distinct contrast in thin sections (0.5 mm) than in thick sections (6 mm). Thus, there is a trade-off between specimen size and image quality. Selection of a specimen as small as is feasible while maintaining the 3D nature of the data for a given application will produce the best results.

We also looked at the effects of the fixation protocol on the autofluorescent images as it has been reported that fixatives affect the fluorescence of cells and tissue (11). We compared our standard 4% PFA fixation to a 1% PFA fixation (Fig. 2). We found that there is a substantial improvement in the OPT image contrast after a 1% PFA fixation compared with a 4% PFA fixation. For example, with a 1% PFA fixation, the layering in the cortex and the striations in the caudate/putamen are more clearly defined and thalamic nuclei can be identified.

In quantitative phenotyping based on structural volume, it is important that there is consistency between samples and between groups of mice. In OPT sample preparation, the brains are first removed from the skulls, dehydrated, and then optically cleared. We expected that this processing might increase variability among samples. Therefore, we compared the variability in OPT brain specimens with the variability typical of fixed-brain MRI specimens, which can be imaged within the skull with minimal processing. Using our standard fixed brain protocol and MR imaging (21), we measured the standard deviation of 63 structure volumes (normalized to the structure size) of the individual structures in the mouse brain. In MRI, where the brain is left in the skull, the standard deviation of the structure volumes (normalized to the mean) ranged from 2 to 16% (n = 10 male C57BL/6J mice, 8 wk old). For OPT samples of the brain (excluding the cerebellum and olfactory bulbs), the normalized standard deviation of the 36 structure volumes was larger and ranged from 6 to 28% (n = 9 male C57BL/6J mice, 8 wk old). Taking the 35 structures common to both the OPT and the MRI atlas, we found that the normalized standard deviation of the OPT samples approximately doubled that of the MRI (based on a linear fit), suggesting the OPT sample preparation does increase variability in structural volume measurements (Fig. 3). To account for this variability in statistical analysis, we computed a scale factor for each specimen and then compared corrected structure volumes. After correction with this scale factor, the normalized standard deviation of OPT brain structure volume was reduced to the range of 2–20% (n = 9 male C57BL/6J mice, 8 wk old). The normalized standard deviation of the corrected structure volumes was comparable to those obtained with MRI (Fig. 3).
Therefore, this scale factor was used for detailed volume measurements for phenotyping the male-female mouse brains to follow.

Visualizing Neuroanatomy With Autofluorescence

We used specimen sizes of ~4 mm × 4 mm × 6 mm and a 1% PFA fixation to investigate which mouse brain structures could be visualized with 3D optical imaging of autofluorescence. Although our results indicate smaller sections of the brain would result in better image quality, we sought to identify structures in larger specimens to view them with the maximal spatial context as a survey of brain anatomy. Our results show excellent detail throughout the brain, and many discrete structures can be identified, some of which are highlighted in Fig. 4. Of note is the detail that is seen in the midbrain where the zona incerta, thalamic nuclei, and the lateral geniculate nucleus are distinguished (Fig. 4). In a 4 mm × 4 mm × 3 mm section with a 1% PFA fixation, the thalamic barreloids in the posteriomedial nucleus of the thalamus are identified on an oblique 2D slice (Fig. 4B).

Of the structures we identified with this method, one of the most beneficial to observe with 3D imaging was layer 4 of the cortex (Fig. 5). Traditionally, the whisker barrel field is studied by removing the cortex, flattening it, and then sectioning for histology (13). Imaging in 3D allows image planes through the cortex to be identified to visualize the large and small whisker barrel fields (Fig. 5A). Furthermore, with 3D imaging, the signal intensities can be mapped onto a curved surface running through layer 4 of the cortex, so that the whisker barrel fields can be represented in their native configuration. This provides a more complete representation of layer 4 in situ and allows for visualization of not only the large and small whisker barrel fields, but also other cortical fields (Fig. 5B). This demonstrates one of the clear advantages of mesoscopic 3D optical imaging over other 2D imaging modalities.

Comparison of Male and Female Brain Anatomy by Autofluorescent OPT Imaging

We sought to identify subtle phenotypic differences in brain morphology using computer-automated analyses of 3D optical images. We first assessed volume differences in the male and female brains by computing the effect size resulting from volumetric comparisons at each voxel (Fig. 6). We found that areas in some structures, such as the basal forebrain and cortex, were larger in females than males, while others, such as areas in the hypothalamus, amygdala, and bed nucleus of the stria terminalis, were larger in males than females. These regions are mapped with separate color-maps in Fig. 6.

In addition to identifying voxel-wise changes, we further evaluated segmented structure volumes in male and female mice. We compared the volumes of 36 different brain regions in the hemispheres of wild-type male (n = 9) and female mice (n = 9). Statistical comparison of structure volumes between sexes after correction by scale factors shows that the bed nucleus of the stria terminalis, globus pallidus, internal capsule, midbrain, stria medullaris, stria terminalis, striatum, thalamus, and zona incerta are all significantly larger in male mice compared with age-matched female mice (Fig. 7). The cerebellum and olfactory bulb were not analyzed for this study.

DISCUSSION

Characterization of the structural organization of the brain requires 3D imaging of the mouse brain at high resolution. Mesoscopic optical imaging techniques, including OPT, allow large specimens to be imaged with ~5 μm resolution. In this study, we demonstrated the use of autofluorescent imaging in the mouse brain for detection of brain morphology phenotypes. These, and other optical methods, will be powerful tools for assessing the structural organization of the brain and alterations that occur during development or as a result of disease.

Our results indicate that specimen preparation is an important step that affects the quality of 3D autofluorescent images. We found thinner sections provided higher-quality images and improved structural detail. Larger specimens, of course, allowed for more complete coverage of the tissue. This creates a trade-off between specimen size and image quality that is an important consideration in phenotyping studies. This trade-off is likely to be apparent in OPT and LSFM but may be less significant in block-face methods, such as HREM, EFIC, and serial two-photon tomography (16).

The fixation method also affects the quality of autofluorescent images. We found that lighter fixation, with 1% PFA, provided greater detail in the mouse brain images than standard 4% PFA fixation. It is possible that other fixatives would also produce a greater autofluorescent signal and improved images. Formalin and glutaraldehyde, for example, have been reported to produce greater autofluorescence than PFA (11) and may be advantageous for retaining autofluorescence. Of course, too
little fixation may compromise specimen quality and increases fragility. This trade-off will be a consideration for all forms of autofluorescence based 3D imaging.

In our study, we used ethanol for the dehydration and BABB for the optical clearing of the specimens. Alternative clearing methods have recently been published. Scale is an aqueous-based clearing reagent that may be compatible with OPT (8). A tetrahydrofuran-based clearing method has also been reported recently for UM imaging (6). Both of these clearing methods will likely be applicable to various forms of 3D optical imaging.

Optical imaging using the autofluorescent signal in the mouse brain provides a unique contrast to structures in the mouse brain. We demonstrated visualization of small discrete structures, including, as an example, the thalamic nuclei. This capability enables phenotyping studies in mouse models where these nuclei may be affected. We also demonstrated a unique visualization of the whisker barrel fields in 3D. When the autofluorescent intensities are represented on a 3D surface through layer 4 of the cortex, the whisker barrel fields, as well as other cortical fields, can be appreciated in their native configuration. This will allow for changes in the whisker barrel fields due to genotype or environmental factors to be assessed quantitatively.

We expect that the autofluorescent OPT imaging results that we present here could also be obtained with alternative 3D fluorescent imaging modalities (LSFM, HREM, and EFIC). In fact, a view of the whisker barrel field on a 2D optical slice has been demonstrated previously with UM in the mouse brain at postnatal day 10 (3). Each of these alternative optical methods has their own relative advantages. Higher-resolution images may be obtained by block-face imaging methods, in which limited optical penetration is overcome by successive sectioning of the tissue (as opposed to optical clearing). OPT is an option that is relatively time efficient, requiring only ~15 min for images at 5–10 μm resolution. This makes OPT an attractive option for high-throughput neuroanatomical phenotyping, as images can be acquired quickly.

A number of mouse models of human disease will have subtle phenotypic differences. To determine if OPT autofluorescent imaging could identify such differences, we examined, as an example, sexual dimorphism in wild-type male and female mouse brains that have previously shown differences using both histology (19) and MRI (21). We found statistically significant differences between male and female mouse brain structures consistent with previous reports: the bed nucleus of the stria terminalis (18), striatum (21), and thalamus (21).
also found the globus pallidus, internal capsule, midbrain, stria medullaris, stria terminalis, and zona incerta to be larger in male mice than female mice. Further investigation of these structures will be of interest. For example, Parkinson’s disease, which results from degeneration of dopaminergic neurons in the midbrain and involves the basal ganglia, is more prevalent in males than females (22); therefore, further studies into the causes of sexual dimorphism seen in the globus pallidus, midbrain, and zona incerta may have relevance to this disease. The globus pallidus has also been reported to be larger in males than in females in human studies (17). We did not observe volumetric differences in the structure volumes of the amygdala and hypothalamus, which have been previously reported to be sexually dimorphic (2). This may be because only a small portion of these structures differ between males and females, while we assessed the volumes of whole structures only. This is supported by our data in Fig. 6, which shows significant changes in discrete regions of the hypothalamus and amygdala by voxel-wise analysis.

The increase in resolution of the OPT and the unique autofluorescent contrast allow for different structures to be visualized with OPT than with other imaging tools, such as MRI, which is also used regularly in our laboratory. The voxel size of our standard ex vivo MRI images is 56 μm, and the voxel size of the OPT images in our sexual dimorphism study is ~10 μm. However, we do observe that the variability is increased with OPT due to the more significant sample processing. This needs to be taken into account when undertaking anatomical phenotyping studies. We found that the volume variability in OPT imaging is approximately double that of the variability in fixed brain MRI imaging, where brains can be imaged within the skull and retain their native configuration. To account for the variability in the sample processing, we determined a scale factor for each mouse that was applied to every structure, resulting in a corrected volume measurement. However, in future studies, it would be better to measure the volume of the whole mouse brain, the volume of the portion being imaged prior to sample processing and the volume of the portion being imaged after processing, to better account for sample processing effects separately from biological variability in overall size. We expect this would help to reduce the variability in OPT data. Despite this correction, it is likely that increased variability due to specimen preparation will require an increase in the number of specimens imaged for phenotyping based on optical methods in which extensive sample processing is necessary. Indeed, we found that...
the cerebellum is a difficult brain structure to image with these methods because the folding of the arbor vitae is easily distorted or damaged during processing. This is likely to be less of a problem with optical imaging methods that do not require dehydration and clearing of the brain, such as block-face methods. Nonetheless, 3D optical imaging, including OPT, enables visualization of neuroanatomy with remarkable detail including anatomic nuclei and structural layers, shows potential for systematic phenotyping, and fits conveniently in the workflow of many research laboratories.

Another advantage of optical imaging, in principle, is the possibility of using fluorescent antibodies or proteins to image specific cell types. Antibody-based imaging has been used in the adult mouse brain (3); however, penetration of antibodies is difficult due to the large size. Antibody imaging has been successfully applied to embryo imaging (20, 26) and in lymph
node imaging (9). The addition of antibody imaging and/or fluorescent protein imaging will allow the underlying causes of anatomical differences to be probed. This stands to provide tremendous improvements to our ability to characterize brain structure phenotypes and their underlying cellular compositions.

In this study, we have shown that 3D optical imaging methods provide a powerful approach to anatomical phenotyping, allowing adult mouse brain anatomy to be visualized over large ROIs and in fine detail. Taking advantage of this, we were able to examine subtle changes in the adult mouse brain, which is useful for neuroanatomical phenotyping mouse models of human disease. This technique can also be applied to study neurodevelopment and plasticity where changes in the brain can be monitored in cross-sectional studies. Optical imaging will provide new insights into the organization of the mouse brain through the process of development and in circumstances of disease, thereby providing new opportunities in a broad range of research disciplines that impact brain health.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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