An Optimized and Detailed Step-by-Step Protocol for the Analysis of Neuronal Morphology in Golgi-Stained Fetal Sheep Brain

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Imaging · Brain development · Imaris · Rapid Golgi · Neuron · Gray matter

Abstract
Antenatal brain development during the final trimester of human pregnancy is a time when mature neurons become increasingly complex in morphology, through axonal and dendritic outgrowth, dendritic branching, and synaptogenesis, together with myelin production. Characterizing neuronal morphological development over time is of interest to developmental neuroscience and provides the framework to measure gray matter pathology in pregnancy compromise. Neuronal microstructure can be assessed with Golgi staining, which selectively stains a small percentage (1–3%) of neurons and their entire dendritic arbor. Advanced imaging processing and analysis tools can then be employed to quantitate neuronal cytoarchitecture. Traditional Golgi-staining protocols have been optimized, and commercial kits are readily available offering improved speed and sensitivity of Golgi staining to produce consistent results. Golgi-stained tissue is then visualized under light microscopy and image analysis may be completed with several software programs for morphological analysis of neurons, including freeware and commercial products. Each program requires optimization, whether semiautomated or automated, requiring different levels of investigator intervention and interpretation, which is a critical consideration for unbiased analysis. Detailed protocols for fetal ovine brain tissue are lacking, and therefore, we provide a step-by-step workflow of computer software analysis for morphometric quantification of Golgi-stained neurons. Here, we utilized the commonly applied FD Rapid GolgiStain kit (FD NeuroTechnologies) on ovine fetal brains collected at 127 days (0.85) of gestational age for the analysis of CA1 pyramidal neurons in the hippocampus. We describe the step-by-step protocol to retrieve neuronal morphometrics using Imaris imaging software to provide quantification of apical and basal dendrites for measures of dendrite length (μm), branch number, branch order, and Sholl analysis (intersections over radius). We also detail software add-ons for data retrieval of dendritic spines including the number of spines, spine density, and spine classification, which are critical indicators of synaptic function.

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The assessment of neuronal morphology in the developing brain using Rapid-Golgi and Imaris software is labor-intensive, particularly during the optimization period. The methodology described in this step-by-step description is novel, detailed, and aims to provide a reproducible, working protocol to quantify neuronal cytoarchitecture with simple descriptions that will save time for the next users of these commonly used techniques.

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Introduction

A vast and interconnected network of one hundred billion cerebral neurons forms complex neuronal circuits within the central nervous system. Neurons are the building blocks of these circuits, connecting one another with their extensions, in both proximal and distal regions of the brain. Analysis of neuron microstructure, including axons, dendrites, and spines, can reveal neuronal wellbeing and structural connectivity. While neuronal morphology varies across brain regions and specific cell populations, fundamentally, the neuron is composed of an elongated axon, short-range branching dendrites, and spines (Fig. 1). These neurite structures are unique to neurons and facilitate cell-to-cell connectivity. During mid-to-late gestation of human fetal development, neuronal projections undergo marked outgrowth of the axon (~20 weeks of gestation to birth), dendrites, and dendritic spines (~24 weeks to 1-year postnatal age), such that network communications are built and functional synapses are established [1, 2].

Dendrites project from the cell body and form complex arbors via dendritic branching, which provide a vast surface area for the integration of synaptic input (Fig. 1a, b) [3, 4]. Dendritic spines are membranous protrusions that extend from the dendritic shaft and provide postsynaptic targets for the axons of afferent neurons (Fig. 1c). Dendritic spines are abundant on excitatory neurons, such as pyramidal cells, and exhibit a variety of morphological characteristics described in simple terms according to their shape (neck length and head width), albeit there is indeed a continuum of spine morphology [5]. Spine morphology is dynamic, which is important for the initiation and maintenance of a synaptic connection [6]. Upon initiation of synaptogenesis, the long and thin filopodia-type spines with a narrow neck and no head are the first to sprout. Filopodia are highly motile and initiate a connection by locating, recognizing, and guiding spines to target axon terminals [7, 8]. The mature spines are termed mushroom and stubby according to their appearance, with an enlarged head, fostering neurotransmitter receptors that ensure stability and synaptic activity [5, 9]. Spine morphology is indicative of functional capacity and, as synaptogenesis progresses, the number of immature spines decreases, and mature spines predominate. Pregnancy complications such as malnutrition, infection, and hypoxia in utero during this critical period of neuronal maturation induce neurological injury by impairing dendrite and spine development, including outgrowth [10, 11], and classification of dendrite and spine morphology is therefore an important tool to characterize the effects of neurodevelopmental compromise.

Characterizing neuronal morphology under normal and abnormal conditions is a cornerstone to developmental neuroscience. Golgi staining was first discovered by Camillo Golgi in 1873, called the "black reaction," and to this day remains a reliable method to assess neuronal cytoarchitecture. The Golgi method is used to visualize the entire cytoarchitecture of an individual neuron on a relatively clear background given that only a low percentage (1–3%) of the total number of neurons are stained. Several iterations of Golgi staining exist, but the most commonly used are the Golgi-Cox and Rapid-Golgi methods [12–14]. Generally, the Golgi-Cox method requires a longer incubation period but produces reliable and evenly stained cells, while the Rapid-Golgi method tends to produce more variable staining but is significantly faster [15].

Both Golgi-Cox and Rapid-Golgi techniques have been utilized in the developing brain [16–18]. However, to our knowledge, the methodology of using Golgi staining combined with image analysis using Imaris (Bitplane) software has not been described in detail, despite being reported in several publications [16–18]. Each combination of Golgi stain and imaging software requires optimization, a process that is time consuming, and requires extensive user intervention. The most reported issue with Golgi staining and analysis is the labor-intensive process of acquiring high-definition images of individual neurons and subsequent analysis of microstructure. Freely available software, such as Fiji (Image J; public domain software from the National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/) and Reconstruct (https://synapsweb.cln.utexas.edu/software-0), rely heavily on manual analysis and user intervention [19], thus increasing the duration of workflow and investigator bias. Investigator bias remains a challenge in the assessment of neuronal morphology with semiautomated software programs. Risher et al. [19] worked with the freely available Image Analysis of Golgi-Stained Fetal Sheep Brain.
available software programs, Fiji and Reconstruct, to streamline Rapid-Golgi analysis and minimize subjectivity and investigator bias for analysis of dendritic spines. The resultant protocol allowed for objective classification of spines using accepted geometric characteristics of neck length and head width measurements to categorize spines [19]. Commercially available software programs feature algorithms with automated and semiautomated functions that help to overcome investigator bias. Automated software programs have spine geometry measurements built in to program algorithms for spine classification [20]. However, although these commercial, automated programs are more likely to be faster, they are also more costly and do not eliminate investigator interpretation and intervention. It is noteworthy that programs such as Imaris are traditionally used with fluorescently stained or cultured neurons, and therefore, analysis with Rapid-Golgi-stained neurons acquired with brightfield microscopy requires extensive optimization.

Here, we provide step-by-step instructions for reliable and reproducible methodology for the acquisition and analysis of commonly used methods of Rapid-Golgi neuronal staining with the use of the powerful and sensitive Imaris software (Filament tracer module). This method-

**Fig. 1.** Fundamental neuron structure, microstructure, and orientation within the hippocampus. 

**a** Schematic of a pyramidal neuron showing (i) basal dendrites, (ii) axon, (iii) soma, (iv) apical dendrites, and (v) dendritic spines. Illustration created with BioRender.com (AC233JVTHX). 

**b** Micrograph of Rapid-Golgi-stained CA1 pyramidal neuron from a fetal sheep brain at 127 days of gestational age. Scale bar, 200 μm. 

**c** Micrograph showing dendritic spines. Scale bar, 10 μm. **b, c** Acquired with ×60 oil objective lens. 

**d** Micrograph of Rapid-Golgi stained hippocampus (×20 air objective lens) and representative schematic of the CA1 subregion of the hippocampus. Scale bar, 1.5 mm (left). Basal dendrites project to the SO, cell body layer is found in the SP, and apical dendrites project to the SR (right). 

**e** Magnified view of CA1 pyramidal neuron revealing orientation of shorter (basal) dendrites and longer (apical) dendrites in the hippocampus. **d, e** Created with BioRender.com (RK233JVNBY). SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.
Method/Results

All animal experiments undertaken in this project were approved by the Monash Medical Centre Animal Ethics Committee (MMCA/2016/62) and conducted in accordance with the National Health and Medical Research Council (NHMRC) of Australia, Australian Code for the Care and Use of Animals for Scientific Purposes. Imaging produces extensive data including dendritic length, dendritic branch points, dendritic arborization (an index of branch complexity using Sholl analysis), number of spines, spine density, and spine classification. We provide a complete workflow from tissue preparation and staining through to image optimization and morphological analysis (Fig. 2), as well as pictorial representation of the basic steps outlined in this protocol (Figs. 3a–f, 4a–e, 5). The methodology described herein is novel, and it is our aim to provide a reproducible, working protocol that will ensure the next users of these techniques can immediately undertake analysis, bypassing time-consuming optimization.

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Tissue Preparation and Tissue Staining

An overview of the equipment and software programs used here is presented in Table 1. Fetal sheep of known gestational age were used in this study. At 127 days (0.85) of fetal gestational age, the ewe and fetus were euthanized with an overdose of intravenous pentobarbitone to the ewe, and the fetus was removed. The whole fetal brain was quickly and carefully harvested from the skull and the brain separated into left and right hemispheres. Tissue preparation and tissue staining were conducted in accordance with manufacturer’s instructions as per the FD Rapid GolgiStain kit (FD NeuroTechnologies, Inc.; PK401 Cell Systems Biology). Tissue and staining optimization were undertaken, and slight modifications were made, which are described in Table 1. Predominantly, instructions provided by the FD Rapid GolgiStain kit were followed (http://fdneurotech.com/docs/1333571253_web_pk401-401a-04042012.pdf). Briefly, the left hemisphere was sectioned coronally at 1–1.5 cm thickness. The region containing the dorsal and ventral hippocampus was washed in milli-Q water and then immersed immediately into the impregnation solution (equal volumes of solutions A and B). Two weeks later (with a change of
solution A/B after the first 24 h), the tissue was transferred to solution C and stored at room temperature for a further 72 h (with a change of solution C after the first 24 h). The sections were then transferred to PBS and cut to 100 μm thickness using a Vibratome (VT1200S; Leica). The FD Rapid-Golgi instructions indicate that a tissue thickness between 80 and 240 μm is an acceptable range to have full penetration of solutions and the manufacturer’s instructions recommend using floating sections. However, as the hippocampus is situated by the inferior temporal horn of the lateral ventricles, it can easily become detached when using floating sections; therefore, in the current study, sections were mounted directly onto slides (Superfrost slides 75 × 50 mm, EPBRJ1800BMNZ; Bio-Strategy) and allowed to dry for a minimum of 24 h.

Up to 20 sections were prepared per animal containing the cornu ammonis 1 (CA1) region of the hippocampus. Slides were first rehydrated in milli-Q water, then submerged (Solution D and E) for 30 s, washed in milli-Q water again, and then coverslipped with #1 thickness coverslips (0.13–0.17 mm, 50 × 50 mm, G450; ProSci Tech) using a water-based mounting agent (Aquatex, #1.08562.0050; Merck). In our experience, we found aqueous mounting media helped to prevent tissue cracks. Coding of the slides was carried out to ensure the investigator completing the analysis remained blinded.

**Cellular Criteria**

Neuronal morphology and function vary across and within defined regions of the brain, and morphology is associated with function [22–24]. Therefore, well-defined cellular criteria are important and should be established prior to image acquisition to determine which neurons are suitable for analysis (Fig. 2, Step 3). Neurons can be excluded at a later stage in analysis if an imaged neuron is found to be incomplicant with the cell selection criteria or has a truncated dendrite.

In the current study, the inclusion criteria for CA1 pyramidal neurons in the hippocampus were based on the previous morphometric studies [16, 25, 26] and were as follows: (i) pyramidal neurons characterized as having a triangular shaped soma with the soma located in the stratum pyramidale; asymmetric dendritic length with apical dendrites projecting toward the stratum radiatum and basal dendrites projecting toward stratum oriens (Fig. 1d, e); (ii) separated neurons with no dendritic overlap; and (iii) apical and/or basal dendrites with complete visibility. The exclusion criteria included: (i) dendritic overlap with adjacent neurons; (ii) truncated neurons (neurons with projections that have been cut off due to the plane of sectioning; see note below); and (iii) neurons with incomplete dye transport (due to incomplete impregnation, which similarly appears as an amputated branch rather than tapering neurites [27]). Examples of truncation and incomplete dye transport are shown in online supplementary Figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000524055).

**Note, truncation of the dendrite occurs from sectioning. Avoid imaging these neurons by selecting neurons whose soma is sufficiently embedded in the thickness of the tissue (i.e., deep to the cut surface).**

The brain region of interest (ROI) was identified at ×2 magnification and neurons that fit the cellular criteria were imaged at ×30 magnification. In the 127-day (0.85 gestation) cohort of animals (n = 7) presented in this paper (shown in Figs. 3, 4), a total of 121 CA1 pyramidal neurons were reconstructed and analyzed. A minimum of n = 10 neurons per animal were analyzed; n = 57 apical and n = 64 basal dendrites. Note that only one animal (n = 5 apical dendrites and n = 9 basal dendrites) was used for the branch hierarchy analysis (Fig. 3i), as this analysis requires an additional extension, and the purpose of these data was to simply showcase the capabilities of Imaris. The number of neurons selected per animal for this analysis was based on the average number of stained neurons present in the CA1 region of the hippocampus that fitted the above criteria for sampling. We did not randomly select neurons, as we wished to image all neurons that fit the cell

![Image Analysis of Golgi-Stained Fetal Sheep Brain](https://example.com/image.png)
(For legend see next page.)
selection criteria. Dean et al. describe a systematic approach to avoid selection bias by randomly selecting every 15th neuron from a sample of preselected neurons [16].

Image Acquisition

In our experiment, large, z-stack images of pyramidal neurons in the hippocampus were acquired on an Olympus BX61 Stereolohger microscope (upright; Olympus, Tokyo, Japan) equipped with an Olympus DP73 color camera (×0.5 lens) and CellSens acquisition software (version 1.14) using a ×60 oil objective lens with 1.42 numerical aperture (NA). A high NA objective, such as 1.42 NA, provided high-resolution images to allow spines to be resolved. An objective with low NA, such as <0.6, would be appropriate if spine analysis was not being conducted. For further reading on optimal image acquisition settings with Golgi, we suggest Mancuso et al. [28]. For most, any microscope with an automated stage and compatible imaging software capable of producing a stitched x, y, z image can be used. Images must be suitable and compatible with the specific imaging program used (i.e., image optimization software and Imaris).

Because high-resolution images generally have a limited field of view, multiple high-resolution images can be constructed into a single, high-resolution image, using the Stage Navigator tool in CellSens. The Stage Navigator constructs a stitched image via multiple image alignment and was used to initially generate a focus map of the brain and subsequently identify the brain ROI (Fig. 3a). To do this, first select the bright-field camera filter and the objective lens with the lowest magnification to obtain the widest field of view. In the camera control window, adjust the exposure time and focus the camera on the tissue, ready for acquisition. Next, in the Stage Navigator tool window, select “Define Overview Area” (this is completed by clicking the “top left” and “bottom right” of your overview area) and click “Acquire Overview.” The automated stage will move and construct the stitched image (Fig. 3a). Once the focus map is obtained, identify the ROI, that is, the hippocampus (Fig. 3b) and change the objective lens to the desired magnification for image acquisition of the neuron, that is, ×60 oil immersion objective lens. Manually focus on the target neuron and image basal and apical dendrites separately (Fig. 3c). Here, the Stage Navigator enables neurons with varying dendritic lengths to be captured by setting the XY position resulting in a stitched image. Z-stack images are acquired to capture dendritic spatial information, and, based on our optimization, z-stack parameters were kept consistent using a predetermined total z-stack range of 25.2 μm and optical section thickness of 0.6 μm, to provide a 43-stack image. The standardized z-stack thickness of 25.2 μm was sufficient to capture all dendritic projections. During optimization, increasing z-stack thickness offered no benefit to capture dendritic depth perception but rather, oversampled the neuron and increased background noise. Further, increasing z-stack thickness dramatically increases overall imaging and analysis time and resulted in very large file sizes. Individual users may choose to adjust thickness between images to capture varying dendritic depths based on individual optimization. Consideration of z-stack thickness may vary between experiments. For example, if only tracing dendrites, a large z-stack thickness with increased optical thickness (i.e., 1–2 μm) might be sufficient. Alternatively, if tracing spines, reducing the z-stack range and the optical thickness (i.e., 0.6 μm) provides better optical resolution and accuracy for tracing spines.

Images were saved as .btif or .tif depending on the file size: .btif for larger images and .tif for smaller images. Images are also automatically saved as the raw .vsi format containing image metadata.

Image Optimization

Imaris is well described for fluorescently stained images but with images acquired with bright-field microscopy, additional steps are required before importing into Imaris. These steps are completed using Fiji (version 1.53c, public domain software from the National Institutes of Health; https://fiji.sc/) [29]. Fiji is a freely avail-

![Fig. 4. Spine quantification and classification.](image)

**Fig. 4.** Spine quantification and classification. **a** Dendrite segment in 2D “Slice” mode showing dendritic spines with clear visibility. **b** False-positive spine tracing, that is, too many spines. **c** Inaccurate spine tracings, with some spines missing, that is, too few spines. **d** Accurate spine tracing. Ascertain accurate spine tracing by cross referencing with an image in 2D, that is, when you count the same number of spines in the respective image. **e** Dendritic segment workflow from image acquisition (scale bar, 10 μm), visualization in Imaris 2D “Slice” mode, and spine tracing and classification. **Note,** the different colour tracings correspond to spine morphology and classification using the “Spine classification” tool in Imaris (i.e. pink is Filopodia, blue is Long Thin, and red is Stubby). **f, h** Apical dendrites, number of spines, and spine density per 10 μm of dendritic length. **g** Basal dendrites number of spines and spine density. **n** = 7 animals, **n** = 35 apical, and **n** = 38 basal. Mean ± SEM.
Fig. 5. Imaris step-by-step workflow. a Basal dendrites in the surpass mode following import. Green arrowhead indicates the icon for the Filament Tracer module. b Step 2/5 2D “Slice” mode needed to measure the soma and, subsequently, spine neck length and head width. Green arrowhead indicates button to access “Slice” mode. c Step 3/5 manually inserts “Starting Point,” that is, soma and “Seed Points,” that is, dendrites. Green arrowhead indicates manual thresholding selection “Starting Points Threshold” and yellow arrowhead indicates manual thresholding selection for “Seed Points Threshold.” d Step 4/5 shows traced dendrites in yellow. Green arrowhead showing manual thresholding selection. For deleting unnecessary branches, yellow arrowhead shows how to complete the sequence. The red arrowhead shows the Filament line you have been working on, and the blue arrowhead shows how to export and save the Imaris file. e An example of the final traced basal dendrites. f Schematic of branch hierarchy. Scale bar, 50 μm.
Table 1. Checklist of the equipment and software programs required to complete neuronal morphological analysis as described in this protocol with potential alternatives and considerations provided [12–14, 21]

| Equipment step                  | used in this protocol                                                                                                                                                                                                 | alternatives and considerations                                                                                                                                                                                                 |
|---------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Tissue staining                 | FD Rapid-Golgi stain kit (FD NeuroTechnologies, Inc.; PK401 cell systems biology)                                                                                                                                                                                               | A full description of the tissue-staining steps can be found in the FD Rapid-Golgi stain kit user manual. Please refer to the text under “tissue preparation and tissue staining” for more detail. Here, we note deviations made for the optimization of fetal sheep brain. Alternative staining methods include Rapid Golgi, Golgi-Kopsch, and Golgi-Cox [12–14]. Published methodologies are available, for example, Golgi-Cox conventions [21]. Commercial kits may be sourced, such as the Hito Golgi-Cox OptimStain™ kit (HITOBIOTEC CORP) |
| Impregnation solution           | Tissue was washed and immersed in the impregnation solution. Effort was made to protect tissue from light, and all tissue-staining steps were performed at room temperature. Following optimization, we found a 2-week impregnation period provided the best staining results. The optimal time for specific tissue might require trial to avoid under- or overstaining dependent on tissue type and size. Following impregnation, tissue was either sectioned immediately or stored at 4°C until ready to be sectioned. |
| Sectioning                      | The user manual recommends using a cryostat, vibratome, or sliding microtome. We did not embed impregnated tissue in agarose/gelatin or freeze the brain tissue. Instead, we directly mounted the brain tissue on the vibratome for sectioning using PBS to wash and/or collect the sections onto slides from the filling chamber. This helped the tissue sections to adhere onto the microscopy slides and prevent cracking when drying. |
| Slide preparation               | The user manual recommends gelatin-coated microscope slides for better adhesion. We used extra-large super frost slides, not gelatin coated.                                                                                                                                       |
| Tissue staining                 | A final solution is used to activate tissue staining allowing visualization of neurons. The staining steps were adapted from the user manual for mounted sections, instead of floating sections. The staining solution mixture was 25% solution D, 25% solution E, and 50% milli-Q water. The user manual suggests 10 min for this step. We found that 30 s was optimal. We did not counterstain. Effort was made to avoid sections from drying out between steps and before cover slipping. Individual tissue optimization is recommended |
| Image acquisition               | Olympus BX61 Stereologer microscope (upright; Olympus) equipped with an Olympus DP73 color camera with ×0.5 lens.                                                                                                                                                               | A bright-field stereologer microscope with an automated stage, extendable x, y axis and z-stack stitching capabilities and equipped with compatible imaging software. Optimal imaging requires an objective with a high NA for high-resolution images |
| Cell imaging                    | CellSens acquisition software (version 1.14)                                                                                                                                                                                                                                    | Any imaging software that is connected to the microscope, and able to produce a compatible image with subsequent steps below may be used.                                                                                     |
| Image optimization              | Fiji (version 1.53c)                                                                                                                                                                                                                                                             | Any imaging program with the following capabilities can be used: (i) rescale pixel to image ratio, (ii) delete channels*, and (iii) crop x, y axis*. *These steps can be completed in Imaris; however, we recommend that it is worthwhile completing beforehand |
| Image analysis                  | Imaris (version 9.2.1)                                                                                                                                                                                                                                                             | This software program extension is required to perform tracing of dendrites and spines with the Filament tracer module. This software program extension is required to perform spine classification following tracing of spines |

*Tissue-staining steps conducted in accordance with manufacturer’s instructions outlined in the FD Rapid-Golgi Stain kit User manual. Any deviations for the optimization of fetal brain tissue are described in further detail in this table.*
able software that can be downloaded and is recommended for this step. Because Fiji is an open-source software (meaning that it is open to members of the public who can use it and add features, expanding Fiji’s capabilities), there are multiple ways to achieve the same command and the steps below provide an example methodology to achieve the requirements. No plugins from separate downloads are required for this section of analysis.

In this step, z-stack images (.btif/.tif) are imported into Fiji. First, the pixel size (i.e., microns per pixel) is adjusted to rescale the image. Rescaling is important to ensure the measurements in the Imaris software are accurate and must be completed prior to import into Imaris. Complete this step by “Analyze” -> “Set Scale.” The pixel size will depend on the specifications of the objective utilized for image acquisition. In this case, the ×60 objective lens has a 0.1481 μm/pixel (i.e., “Distance in pixels” input “1”; “Known distance” input “0.1481”; “Pixel aspect ratio” input “1”; “Unit of length” input “μm,” with the resulting scale set at 6.7522 pixels/μm). Once the image has the correct scaling, configuration of the image will need to be cropped on the XY axis to discard any unnecessary field of view that does not contain dendrites associated with the neuron of interest. This will result in an image of apical or basal dendrites only. Further, because images are acquired with a color camera, the RGB image consists of three channels and unnecessary channels will need to be deleted. This can be completed with the same command to crop the XY axis, and 2 of the 3 channels of the RGB image should be deleted. The cell area of interest can be cropped by utilizing the “Rectangle” tool and selecting the ROI (either the apical or basal dendrites, with the soma). In this way, the x- and y-axis can be cropped, as well as deleting the unnecessary channels, in the same command. Once the ROI is selected (Fig. 3c), select “Image” -> “Duplicate.” Select check box for “Duplicate hyperstack” and delete 2/3 channels (i.e., input “1” for “Channels”). Ensure that all frames are selected, in this example there are 43 images configuring the hyperstack. This automatically inverts the image, and the output image then resembles Figure 3d. Alternatively, the channel of interest can be selected (refer to step 2 below under, “dendrite and spine analysis using Imaris-Filament Tracer”), or channels can be deleted (refer to “Imaris import,” below) in Imaris. However, in our experience, deleting the unnecessary channels prior to import results in a smaller file size and a smoother and faster function in Imaris. The image can be saved as .tif or .btif (depending on the size of the file). The image sequence is now ready to be imported into Imaris.

**Imaris Import**

In this workflow, Imaris 9.2.1 is used. Open Imaris and select “FilamentTracer” and “Imaris XT” extensions upon start up. Create a new “Group” in the Imaris “Arena” and drag the .btif or the .tif file to import into Imaris. The group can be labeled according to your experiment. It is possible to save images to the “Arena” following analysis. However, we recommend that files are saved to a separate hard drive as to not consume space on the computer drive, which might slow the functioning of the program during analysis.

Double click to open image and view in the “Surpass” mode (Fig. 5a). The image will need some adjustments before commencing analysis with the Filament tracer module. If the Z-stacks are represented as time (i.e., the image can be played as a movie), switch the frame from time to Z-stacks by navigating to the top tool bar and performing “Image Processing” and select “Swap Time and Z….” The Filament tracer module is not compatible in the movie mode, so this step is essential. Next, ensure the contrast signal is analogous with the neuronal filament by inverting the image. This can be completed by navigating to “Image processing” -> “Contrast change” -> “Invert” -> “All channels” -> “Apply.” Additional edits to render the image ready for analysis include: (1) “Edit” -> “Delete channels” (alternative method if not done in Fiji as detailed in “Imaging processing” above), (2) “Image processing” -> “Contrast change” -> “Normalize layers,” and (3) “Image processing” -> “Contrast change” -> “Linear stretch” -> “Apply.” It is normal for Golgi-stained images to have some background noise (i.e., artifacts, stained vessels, glial cells, and cross sections of stained neurons), and this can be accounted for, without altering the metadata of the image by going to “Image processing” -> “Thresholding” -> “Baseline subtraction” -> “Apply.” The baseline subtraction process does not need to be standardized across all images as it does not alter the metadata in the image, and it simply aids visualization of the dendrites. With this tool, you can manually and incrementally reduce the background noise, taking care not to miss any smaller details on the filament, especially the dendritic spines. Cross check that none of the smaller dendritic

**Dendrite and Spine Analysis Using Imaris-Filament Tracer**

The extensions required for this protocol include product “FilamentTracer” and “Imaris XT.” Details are described in the Imaris Reference manual (version 9.2.1, http://www.bitplane.com/download/manuals/ReferenceManual9_2_0.pdf).
spines have been lost in 2D slice mode or have the same image open in Fiji for comparison. Alternatively, this can be achieved by “Edit” -> “Image adjustment” and adjust only the minimum and maximum values, taking care not to adjust the gamma (as to not alter the metadata). Save your image to an external hard drive using the “Export” function and continue analysis with the Filament tracer module (the term filament in Imaris refers to all neuronal projections including the dendritic shaft and dendritic spines).

Filament Tracer Module for Dendrite Tracing and Dendrite Spine Analysis

The image is now ready to perform analysis with the Filament tracer module, which is used to trace the dendrites and dendritic spines. Apical and basal dendrites are analyzed separately. There are 5 steps to complete tracing of the dendrites and an additional 2 steps to trace spines, if this option is selected. Click the “Add new Filaments” (green arrowhead Fig. 5a) on the software wizard and a new line will appear titled “Filament 1.” Click on this and proceed with the wizard that appears on the bottom left of the screen.

Step 1/5 Algorithm. Keep “Favorite Creation Parameters” as “*Default*” and “Algorithm Settings” as “Auto-path (no loops).”

Note, this analysis is not automated, further stressing the importance for the investigator to be blinded, as it relies on investigator manual input to complete filament tracing.

Uncheck the box “Segment Region of Interest.” Theoretically, you can use this option (“Segment Region of Interest”); however, in this workflow, this has already been completed in the image optimization step. Further, due to the background noise in the image, the diameter tool cannot be utilized (compatible with fluorescently stained neurons). Fluorescence staining is required to measure dendrite thickness/volume with Imaris as it is not possible to remove background noise within the Golgi image without altering image metadata.

Step 2/5 Dendrite Points Diameter. For “Select Source Channel” select channel 1. “Starting point” refers to neuronal soma (or neuron cell body). To measure the largest diameter, switch to “Slice” mode (top panel) for 2D visualization of the hyperstack (Fig. 5b green arrowhead). Scroll through the stacks and when the soma is in focus (left panel should be around the middle of your stack) measure the longest diameter with the “Line” tool (Fig. 5b yellow box). Record this as the “Starting point” (i.e., 25.5 μm). The “Seed Point” is used to trace the dendrites and is kept consistent at 1 μm for all images. Although the width and diameter of the dendritic shaft vary within and between neurons, in our experience 1 μm works very well to trace the dendrites consistently.

Step 3/5 Classify Dendritic Points. The program will automatically plot “Starting Points” and “Seed Points” based on the values you have provided, and the metadata held within the image (visualized as the contrast signal of the image). However, as the Golgi image contains background data and contrast signals from sources other than the neuron itself, the algorithm built within the software is unreliable, offsetting the accuracy of the automated control. Therefore, removal of these entries is required to enable manual entry of both the “Starting Points” and “Seed Points” for manual tracing of the dendrites.

The “Starting Points Threshold” can be manipulated in the Filament tracer wizard to remove the automated “Start Points.” Using the manual thresholding option, add a number at least one value larger (Fig. 5c green arrowhead) than what is generated in the box next to it on the right (these values are automatically generated based on your image). The automatically generated “Start Points” will disappear.

Similarly, the “Seed Points Threshold” can be changed to remove the officious number of “Seed Points.” Simply insert a very large number (i.e., 100,000,000,000,000,000) in the respective field in the Filament tracer wizard (Fig. 5c yellow arrowhead). You can adjust the “Remove Seed Points Around Starting Point(s)” if you have dendrites close to the neuronal soma.

The image is now clear of automatically generated entries and is ready for manual entry to trace dendrites. Ensure you are on the “Select” pointer (Fig. 5c red arrowhead) and insert the “Starting point” by pressing the shift key and right click on the neuron soma. There should only be one “Starting Point” per image. At each dendrite branch point (node) and terminal point, insert a “Seed Point” by holding the shift key and left click. The last “Seed Point” in the sequence will indicate the dendrite terminal. There are a minimum number of one “Seed Points”; however, depending on the number of dendrites, there is likely to be many more.

Note, a “Seed Point” indicates a point of bifurcation or a dendritic “branch.” However, sometimes additional “Seed Points” are required for images with a low-contrast signal of the dendrite. This will require many additional “Seed Point” entries along the shaft of the dendrite to accurately trace the dendrite and ensure all dendrites are captured. Additional “Seed Points,” required to trace the image, may slow down the analysis causing the program to take longer to complete (i.e., don’t overdo this if you can...
avoid it) and additional “Seed Points” will also create erroneous branches (i.e., a false positive). If this occurs, unnecessary branch/s can be deleted after the dendrites have been traced (described below, deleting false branches), but will need to be completed before spine analysis. This is important because dendrite branch number is one of the main morphological parameters of interest and will distort other measurements such as summated dendritic length, resulting in inaccurate quantification.

When the “Starting Points” and “Seed Points” have been inserted, click next. This step can take a while, depending on the size of your neuron/image and the amount of “Seed Points” inserted.

**Step 4/5 Dendrite Diameter.** When you reach this step, you will need to make sure the tracings of the dendrites are accurate. Lower the threshold to the minimum (Fig. 5d green arrowhead) so you can see the tracing generated (Fig. 5d yellow tracing). Assess the tracings made, if the tracing of the dendrite is not accurate, you may need to repeat step 3/5 two to four times to ensure the tracing is accurate. To do so, use the blue back-arrowhead and apply changes to the “Seed Points” entries, as necessary. When you are confident that the tracing is accurate, proceed to the next step ensuring the “Appropriate Circle of Cross Section Area” checkbox is selected (this is important for Sholl analysis). If you do not wish to trace dendritic spines, click the green double-arrowhead button to conclude the sequence. If you do wish to trace dendritic spines, press the blue arrowhead (forward) button.

*Note,* the dendrite diameter function is not compatible with Golgi images and the data generated from this function is disregarded.

Deleting Branches
At this stage, when the dendrites are (accurately) traced, you can check to see if any false branches have been generated and delete accordingly by completing the following steps. First, complete the wizard using the green double-arrowhead button located at the bottom left of the Filament tracer wizard (Fig. 5d yellow arrowhead).

*Note: This is a good opportunity to save your progress by exporting the file to a separate hard drive or to the computer (.ims format)*.

Click the Filament you have been working on in the top wizard box (Fig. 5d red arrowhead “Filaments 1”) and select the “Filter” tool in the filament tracer wizard below (torch icon). Here, you can highlight the entire filament by clicking “Add” with the “Filter Type” and select “Dendrite Area.” Increase the threshold to the maximum value so that all dendrites are highlighted. Delete the unnecessary branches by using the “navigate” tool to zoom in closely to the dendrites and, if an unnecessary branch is found, switch to the “Select” tool, and press the “control” key and left click on the false branch. When all the false branches are deleted, press “Duplicate Selection to new Filaments.” A new Filament will pop up in the above wizard, titled “Filaments 1 copy.” This contains the final dendrite tracing (Fig. 5e), which is the conditioned filament used to extract data about the dendrite and is used to complete spine analysis.

**5/7 Spine Points Diameter.** Once the dendrites are traced, if you are following through directly from step 4/5 Dendrite diameter, simply check the box “Detect Spines” and select the appropriate channel (there should only be one channel). If you have conditioned the dendrites by deleting unnecessary branches, select the Filament you would like to perform the spine analysis on (i.e., “Filaments 1 copy”) and click on the “Creation” tab in the Filament tracer wizard below (wand icon). Under “Rebuild,” select “Rebuild Spine.” This will add 2 steps to the workflow, increasing /5 to /7 steps (step sequence matches what is generated in Imaris software program).

There are two input values needed to detect spines, “Seed Points” and “Maximum Length.” “Seed Points” refers to the minimum width of the spine head in micrometers, and “Maximum Length” refers to the maximum length of the spine neck in micrometers. As done for measuring soma longest diameter, measuring spine head width and neck length can be completed in 2D “Slice” mode. Using the “line” tool in “Slice” mode, measure several spines, in several regions, to get an accurate idea of spine morphology. Once you have determined the values for “Seed Points” and “Maximum Length,” proceed to the next step of the Filament wizard.

**Step 6/7 Classify Spine Points.** The software program will ask you to set the “Seed Point threshold.” At this step, several points will be automatically generated (small blue spheres), which represent a spine to be traced, based on the values from the previous step (5/7). This is difficult to determine by eye without a reference image, and because visualization of the spines is clearer in 2D, we recommend you open the same image in a separate window, for example, open in Fiji. Scroll through the 2D stacks while cross-checking the number of blue spheres in Imaris. Sample several regions while adjusting the threshold in Imaris. The number of spines counted on the dendritic shaft in 2D should reflect the number of “Seed Points”/blue spheres, generated in Imaris. Blue spheres will be generated spanning across the entire image. Only the spines that correspond with the values inserted in the previous step (5/7),
that is, “Seed Points” for maximum spine head width and “Maximum Length” for maximum spine neck length, will be traced. When the number of spheres counted along the dendritic shaft in Imaris corresponds with the number of spines counted (by eye) in the 2D image, click next.

Steps 5/7 and 6/7 may need to be repeated up to four times to accurately trace dendritic spines. If so, this will require you to step back (blue backward arrowhead) and adjust the values for “Seed Points,” “Maximum Length,” and “Seed Point threshold” accordingly. Tip: keep note of the values inputted so that you can track your tracing to find the best fit. If false-positive spines have been traced too far from the dendrite, the “Maximum Length” is too long (example shown in Fig. 4b). Similarly, if the spine tracings are too dense, the “Seed Points” width is too low and the “Seed Point threshold” value is too low (i.e., will need to be increased, example Fig. 4b). If the spines are underrepresented as shown in Figure 4c, you will need to adjust for this also. Also noteworthy, some images might not be of satisfactory quality to run the spine analysis, particularly if there is more background staining than usual, cracked tissue, or dendrites with a high degree of varying dendritic shaft width. In this case, exclude the image from spine analysis. When the spines have been traced accurately (Fig. 4d), click the green arrowhead at the bottom of the wizard to move to the last step.

Step 7/7 Spine Diameter. When the spines are accurately traced and there is no need to reenter data, complete the final step “Spine Diameter” by manually reducing the threshold to the lowest value and under “algorithm” select “Appropriate Circle of Cross Section Area” (this is important for the Sholl analysis). Complete the analysis with the green arrowhead button.

Spine Classification
The “Imaris XI” extension and MATLAB (R2019a Inc.) are required to run the spine classification function. Ensure the desired filament (the filament that has the traced spines) is selected on the left toolbar (i.e., “Filaments 1” or “Filaments 1 copy”) and select the “Tools” icon (cog wheel icon). You will see a list of available extensions. Click on “Classify Spines.” Geometric values for the classification of Stubby, Mushroom, Long Thin, and Filopodia spines are provided. Keep the default settings and click “Classify Spines.” A folder sequence will appear under the selected Filament for each spine classification. These are represented on the image and are indicated by their respective colors.

Save the Imaris file using the export function (Fig. 5d blue arrowhead) and save to the computer or hard drive for backup and future reference. It is worthwhile saving the .ims file because once the filament is traced, there are multiple tools and extensions within Imaris that can be used to further analyze and extract information about different morphological characteristics (such as Branch Hierarchy, Fig. 3f and Convex Hull, Fig. 6).

Data Retrieval and Morphological Indices of Interest
Once the filament has been traced and .ims file saved, statistics can be saved in Excel format (.xls, .csv). Select the desired filament (i.e., “Filaments 1” or “Filaments 1 copy”) and click the “Statistics” tab (graph icon). Statistics can be extracted several ways depending on the morphological characteristics (i.e., dendrites or spines) and parameters of interest. This step-by-step protocol has provided the instructions to obtain quantitative statistics for dendrite length (μm), branch number, Sholl analysis (intersections over radius), spine number, and spine density (per 10 μm of dendritic length), as seen in Figure 3g–i, k, for dendrites, and Figure 4f–i, for spines. To export data for the dendrites, select “Detailed” -> “Specific Values” -> “Export All Statistics to File” and save as .xls or .csv. Within the .xls or .csv file, navigate to “Dendritic length” to calculate summated dendritic length (μm), “Filament No. Dendritic terminals” for a count of the number of branch points and “Filament No. Sholl Intersections” for Sholl analysis. Sholl analysis is useful for providing a measurement of dendritic arborization and complexity.

Note: The distance between concentric rings for Sholl analysis (Fig. 3k) can be changed by “File” -> “Preferences” -> “Statistics” -> “Spheres.” In our analysis, we used 20 μm for the distance between concentric rings.

Data regarding spines can be downloaded individually by selecting the folder for each subclassification, i.e., Stubby, and selecting “Statistics” -> “Detailed” -> “Average Values” -> “Export Statistic on Tab Display to File.” The total number of spines (for the respective subclassification) is indicated by “Dendrite No. Spines” (under the column “sum”), and the spine density is indicated by “Dendrite Spine Density” (under the column “mean”) and is calculated per 10 μm of dendritic length.

Tutorial Data Set
An example of one image represented in the included data set (Figs. 3, 4) can be freely accessed and downloaded at https://tinyurl.com/GolgiTutorialSet. This example of a basal dendrite has been analyzed with the steps outlined above. With access to the required software systems, this image can be used to familiarize yourself with the steps described in this protocol. The example files include...
the RGB image (BasalExample_Preoptimization.btf), optimized image (BasalExample_Optimized.tif), traced image (BasalExample_traced.ims), and associated excel spreadsheets (.xls). Data from three tabs in the BasalExample_Dendrites.xls file are shown in this protocol including “Dendrite Length” for summated dendritic length, “Filament No. Dendrite Terminal” for branch number, and Filament No. Sholl Intersections for Sholl analysis (highlighted in yellow). Data used for dendritic spine analysis are shown in the files titled BasalExample_Filopodia.xls, BasalExample_Long Thin.xls, BasalExample_Mushroom.xls, and BasalExample_Stubby.xls. The data values for total number of spines (sum) and spine density (mean) are highlighted in yellow.

Tutorial images have been provided in a compressed format, for example purposes only. Always keep images in the raw format (.vsi) or .tif/.btif for image analysis. VSI and TIFF files are recommended to avoid compression as this will result in a loss of pixels during file compression, leading to inaccuracies.

Statistics
Example data extracted from CA1 pyramidal neurons of the hippocampus from 127-day (0.85) gestation fetal lambs are provided in Figures 3 and 4. The purpose of these descriptive data sets is to showcase the morphological indices that can be analyzed with the methodology described in this paper. Data sets were analyzed using GraphPad Prism (version 9.3.0). Dendritic morphology for apical and basal dendrites is provided in Figure 3g–i, k, including dendrite length (μm), branch number, branch order, and Sholl analysis (intersections over radius). Note that the branch order requires an additional extension, “Branch Hierarchy,” not detailed in the methodology here but has been included to demonstrate the vast utility for using Imaris with Golgi stained images. Dendritic spine data including spine number and spine density (per 10 μm of dendritic length) can be seen in Figure 4f–i. Data are presented as mean ± SEM.

Discussion
The combination of Golgi staining and advanced image analysis provides gold-standard methodology for tracing and cytoarchitectural analysis of individual neurons [12, 13]. Unlike any other staining technique, Golgi reveals the entire microstructure of an individual neuron. Our step-by-step guide for the use of Rapid-Golgi staining and image analysis using Imaris software is reliable and feasible for quantification of neurons in the developing ovine brain. It is our aim that future researchers using
Numerous methods exist for quantification of neuron number, morphology, and connectivity. Nissl staining works best with large-bodied neurons and may not detect those with small bodies or interneurons [30]. Immunohistochemistry can also identify neurons and their projections with markers including NeuN for cell bodies, microtubule-associated protein 2 (MAP2) for dendrites, synaptophysin for presynapses, and postsynaptic density protein 95 (PSD95) for postsynaptic terminals (or dendritic spines). These do not, however, offer the unique advantage of Golgi in staining the entire neuron structure including the axons, dendrites, and spines. Electron microscopy remains the gold standard for visualizing synaptic morphology by providing a complete 3D reconstruction of a synapse using immunogold labeling [31]. The challenges of overcoming low-throughput electron microscopy imaging has been addressed elsewhere [31], but here we describe an alternative method using conventional light microscopy for measuring neurite and spine morphology. Further, great advances have been made in whole brain imaging, accelerating our ability to detect and quantify structural and functional connectivity in the human brain noninvasively as well as experimentally (including the organization of neural pathways and network architecture) [2]. Human neuroimaging, including MRI, diffusion tensor imaging, tractography, and diffusion MRI for white matter analysis, can be used to identify macroscopic differences between healthy and pathological brains. However, the ability to accurately quantify microscopic neuronal morphology in connection with synaptic plasticity will always be of critical interest to neuroscientists.

The advantages of Golgi staining for assessment of neuronal morphology are clear, and evolution of image analysis has resulted in greater sensitivity and reduced analysis time [13, 14, 19]. However, it remains that compared to other neurostructural visibility techniques such as immunohistochemistry and MRI, Golgi analysis is a time-consuming task. Here, we aimed to expedite this process. Using our protocol, the average time for image acquisition is ∼3 min, optimization up to ∼2 min, while exporting the file, and image analysis including tracing dendrites requiring ∼5–10 min. The majority of time will be spent on computer processing, reflective of cell complexity. A neuron with little dendritic arborization might take only 20 min for processing and dendritic tracing, but a complex neuron can take 1–2 h. Analyzing dendritic spines requires little manual input, around 1–2 min to insert the necessary values, and computer processing will vary depending on spine density.

Pregnancy compromises such as fetal growth restriction and preterm birth (independently) have significant consequences for brain connectivity demonstrated with neuroimaging [11, 32, 33]. Importantly, preclinical studies enhance our ability to study, measure, and understand changes at the cellular and subcellular levels, illuminating altered brain development and neuropathology [17, 25]. To date, published papers in the developing brain have utilized Rapid-Golgi staining together with several different software programs. Risher and colleagues provide methodology using freely available software programs for manual interpretation of neuron morphology and spine characterization in the immature mouse brain [19]. Dean and colleagues [16] used Rapid-Golgi with Neurolucida to quantify neuronal morphology in a fetal sheep model of cerebral ischemia and show impaired expansion of the dendritic arbor and a significant reduction in spine density following ischemia. Preclinical models of fetal growth restriction have used the Rapid-Golgi method with ImageJ (with ImageJ plugins for neuronal morphological analysis) [17, 34], showing altered morphology of CA1 pyramidal neurons and a significant reduction in dendritic length [17]. Interestingly, an unexpected increase in spine density was seen in a guinea pig model of fetal growth restriction [17]. However, CA1 pyramidal neurons of the hippocampus have shown to be vulnerable to injury when exposed to adverse antenatal environments [35–37]. Further, using this technique (Rapid-Golgi with ImageJ), Gilchrist and colleagues recently published findings that a rat model of placental insufficiency did not alter apical dendrite length, number of intersections, and Sholl analysis of dentate granule cells in the hippocampus [34]. Prasad and colleagues [25] showed neuronal dysmaturation in the early postnatal period of a preterm rat model of LPS-induced inflammation, combining Rapid-Golgi and Neurolucida techniques. Critically, altered neuronal development can reduce the strength and regularity of electrical impulse, compromising signal transduction and dendritic spine maturation [38]. Human postmortem studies have also used Rapid-Golgi and Neurolucida for analysis of neuronal microstructure [39, 40]. Studies in human postmortem tissue have documented the developmental trajectory of hippocampal and cortical neurons from midgestation to childhood, whereby somal growth and intensive dendritic arborization occur predominately in the second and third trimesters of pregnancy (stabilizing after 38 weeks of gestation) [39–41], and spine development starts in the third trimester and...
continues throughout childhood [39, 41]. The findings from such studies provide invaluable information on the normal development of neurons as well as pathological changes in response to compromise.

Golgi-stained tissue can be analyzed with various software programs [16, 17, 19]. Similarly, Imaris software is compatible with various staining techniques, including immunofluorescence, immunohistochemistry, and Golgi [42–46]. The tissue stain selected, for example, Golgi or immunofluorescence, will dictate the type of microscope required for imaging and the subsequent steps needed to optimize and complete analysis. For example, Golgi-stained tissue requires bright-field microscopy and fluorescently stained tissue can be imaged with wide field epifluorescence as well as confocal microscopy. In turn, image format will be determined by the method of image acquisition and microscopy specifications, which will determine Imaris-compatible functions. Confocal images of fluorescently stained tissue together with Imaris analysis have been used to analyze spines on neurons in culture and in situ [47, 48], providing quantifiable 3D volumetric spine measurements [47]. Alternatively, light microscopy (used with Golgi) generates an RGB image whereby only one channel is used for image analysis in Imaris using the filament tracer module. Specific microscopy criterion for image acquisition varies depending on tissue staining and available resources (i.e., microscope and software programs; see Table 1). Due to the large size and irregular shape of neurons, a microscope with an automated stage is required, enabling an extendable XY axis and precise Z steps. This is particularly so if the neurons are very large, as they have been documented to be in the human brain and large animals [49]. Further, Golgi sections are cut comparatively thick, at 100–200 μm (compared to 5–10 μm used for immunohistochemistry), thus requiring a microscope with z-stack acquisition capability (a z-stack image is also needed for analysis in Imaris) and a high NA oil immersion objective. Two-dimensional images and z-stack images can be used in Fiji for analysis of neuronal morphometry such as neuron soma area and spines [19].

Imaris software is advantageous for neuronal morphological analysis because automated functions enable the detection, tracing, and measurement of critical neuronal components, analyzing structure at a per-cell basis with the added ability for segmentation of subcellular components. With this modality, dendrites, dendritic spines as well as their organization, structure, and complexity can be quantified and measured in a multitude of ways with just a few simple steps. Importantly, Imaris gives the possibility of removing unwanted artifacts present in the tissue, otherwise obstructing the field of view, and removing nonneuron components in the image (Fig. 3c red arrowhead). As a result, the neuron is able to be cleanly traced for morphological analysis (Fig. 3e). Therefore, while Imaris is commercially available and more costly (requiring a license) than freely available software, it can remove nonneuronal contrast signals within the image and provides unbiased and efficient analysis of neuronal microstructure.

The limitations of this methodological protocol, further to cost, are that despite advances in staining, image acquisition, and analysis, the technique remains a relatively time-consuming process. A large sample size of the number of neurons is required in any tissue sample to be representative of the neuron population of interest and to provide adequate statistical power where differences between groups are quantified. A further limitation is that traditionally z-stacks are not performed using brightfield since the projected z inverts the image, which creates shadows from the layers above and the resulting image may miss overlapping dendrites and spines. This is an inherent limitation that may lead to an underestimate of dendrites and spines across all neurons sampled, and iterates the importance of acquiring multiple replicates to reduce variability.

In this work, we provide a detailed step-by-step easy to follow protocol for Golgi stained fetal brain tissue using Imaris software for image analysis. It is our aim through this protocol paper to reduce the experimental time required to optimize this technique for any researchers setting out in their Golgi journey. We utilized hippocampal sections collected from fetal sheep at 127 days of gestational age, approximately equivalent to 34 weeks of gestational age in humans [50], or preterm neurodevelopment. However, it is in our experience that this methodology is feasible across gray matter regions from mid-to-late gestation and into the neonatal period (unpublished observations). Future studies to determine whether antenatal compromise adversely alters development of neuronal morphology, using the techniques described here, are warranted. This detailed protocol overcomes the challenges in obtaining morphometric values at the neuron level in the developing brain and removes the need for protocol optimization for the next user of this methodology.

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Statement of Ethics

All animal experiments undertaken in this project were approved by the Monash Medical Centre Animal Ethics Committee (MMCA2016/62) and conducted in accordance with the National Health and Medical Research Council (NHMRC) of Australia, Australian Code for the Care and Use of Animals for Scientific Purposes.

Conflict of Interest Statement

Dr. Carina Mallard is an Editorial Board member of Development Neuroscience. Further to this, the authors of the paper entitled “An Optimized and Detailed Step-by-Step Protocol for the Analysis of Neuronal Morphology in Golgi-Stained Fetal Sheep Brain” have no competing interests to declare.

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