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Improved DiOlistic labelling technique for neurons *in situ*: Detailed visualisation of dendritic spines and concurrent histochemical-detection in fixed tissue

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ABSTRACT

DiOlistic labelling is a robust, unbiased ballistic method that utilises lipophilic dyes to morphologically label neurons. While its efficacy on freshly dissected tissue specimens is well-documented, applying DiOlistic labelling to stored, fixed brain tissue and its use in polychromatic multi-marker studies poses significant technical challenges. Here, we present an improved, step-by-step protocol for DiOlistic labelling of dendrites and dendritic spines in fixed mouse tissue. Our protocol encompasses the five key stages: Tissue Preparation, Dye Bullet Preparation, DiOlistic Labelling, Confocal Imaging, and Image Analysis. This method ensures reliable and consistent labelling of dendritic spines in fixed mouse tissue, combined with increased throughput of samples and multi-parameter staining and visualisation of tissue, thereby offering a valuable approach for neuroscientific research.

1. Introduction

Rooted in the neuron doctrine, which describes neurons as discrete, individual units with specialised functions, the intricate relationship between the structure and function of neurons stands as a fundamental concept in neuroscience [1]. Dendritic spines, small protrusions on neuronal dendrites, serve as vital post-synaptic sites for receiving inputs from other neurons. These structures are dynamic, undergoing changes in number, size, and shape – a phenomenon closely linked to synaptic plasticity, a key mechanism underlying learning and memory [2,3]. The continual modification and refinement of dendritic spines are crucial in shaping neuronal networks, particularly evident during developmental network refinement through microglial engulfment of synapses, termed synaptic pruning [4–6]. However, disruptions in this balance of synaptic turnover are associated with various neurological disorders and neurodegenerative diseases such as Alzheimer's disease (AD) [7,8]. This underscores the need for effective methods to visualize and quantify changes in dendritic spines in developmental, healthy and pathological contexts. For the purpose of detailed analysis of these critical neuronal structures, DiOlistic labelling, stands out as a particularly effective technique [9,10].

In 2000, Gan *et al.*, introduced the concept of multicolour DiOlistic labelling for the rapid labelling of neurons in the brain and retina, using ballistic delivery of lipophilic dye-coated particles [9]. This technique presented a significant advance in the analysis of the neuronal connectome. Its ability to achieve Golgi-like labelling with multiple colours significantly advanced the study of neuronal connectivity since it allowed the disambiguation of adjacent neuronal structures, which were either unlabelled or labelled with different dye combinations. Since the inception of the technique [9], optimisation of the DiOlistic labelling have been described and rapidly adopted to characterise the diverse morphologies of neurons [11] and dendritic spines both *in situ* [12–15] and in *in vitro* studies [10,16,17]. DiOlistic labelling is independent of the cell's physiological state, unlike transgenic reporter lines, making it potentially more effective for labeling in diseased tissue [18].

While current DiOlistic methodologies have provided significant insights into the construction and dynamics of dendritic structure in general and dendritic spines in particular, relating measurements in the

Abbreviations: AD, Alzheimer's disease; eGFP, enhanced green fluorescent protein; ROI, Region of Interest; ETFE, ethylene tetrafluoroethylene.

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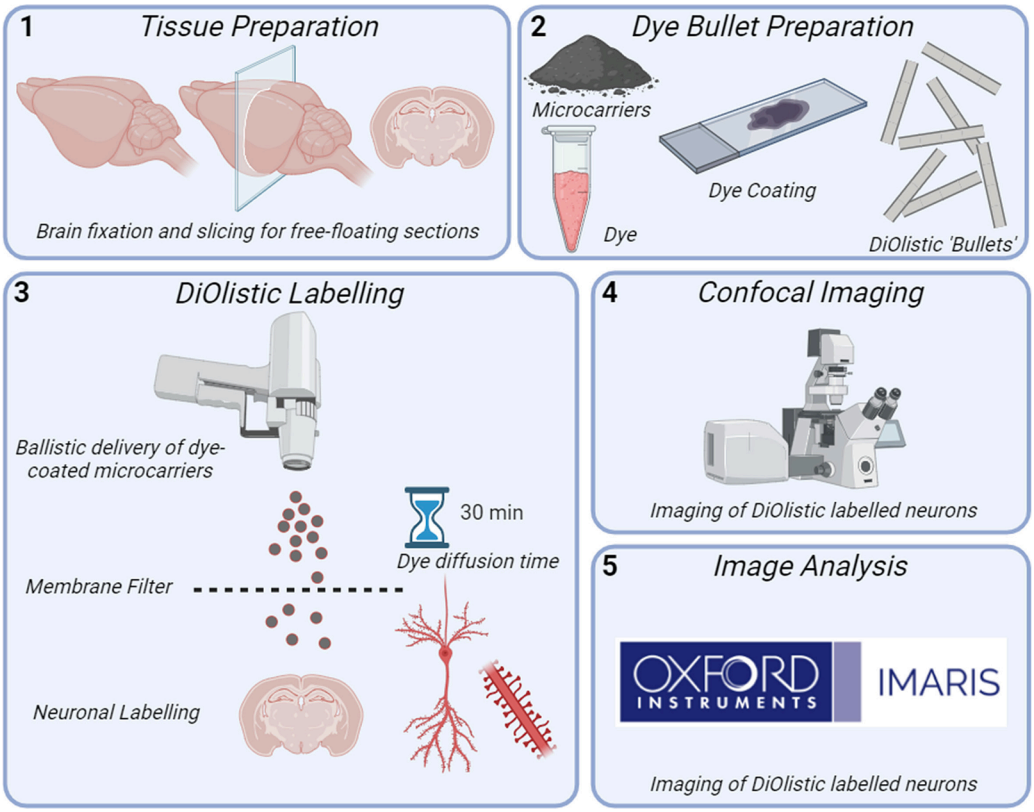

Fig. 1. Stages of DiOlistic Labelling in Fixed Mouse Brain Tissue. 1) Tissue Preparation: Mouse brain tissue is fixed upon collection and sectioned for free-floating slices suitable for DiOlistic labelling. **2) Dye Bullet Preparation**: Tungsten microcarriers are coated with lipophilic carbocyanine fluorescent dyes to create DiOlistic bullets. **3) DiOlistic Labelling**: The ballistic application of dye-coated microcarriers unbiased and random labels neurons within the tissue sections, following dye diffusion. **4) Confocal Imaging**: Neurons labelled with the fluorescent dye are imaged using high-resolution confocal microscopy to visualise their morphology. **5) Image Analysis**: Imaris software facilitates 3D reconstruction and analysis, yielding detailed data on dendritic and dendritic spine structures. Figure created with BioRender.com.

context of neurons, glial and vascular changes in development and disease have been confounded by the difficulty of achieving reliable co-labelling. We have addressed these limitations by developing and refining a reliable fixed tissue analysis approach. By enhancing the DiOlistic labelling technique and optimising subsequent imaging and analysis processes, we offer a more comprehensive, scalable, collaborative and precise approach to understanding the complex role of dendritic spines in neural function in healthy and pathological contexts.

In the course of using the DiOlistic labelling technique [19–23] to study dendritic changes in models including Alzheimer's disease, we have significantly modified the original protocol [9] to yield large number of labelled cells in fixed tissue. Carbocyanine dyes were classically used in unfixed tissue but the degree of labelling was highly variable [9,14,15]. A major problem being that large amount of dye, usually as crystals needed to be inserted, resulting in heavy labelling of cells – to the extent that dendritic analysis was impossible. Even so, these methods were effective for the purpose of axon tracing [24]. DiOlistic labelling is best optimised for live, freshly dissected tissue samples. However, the limited availability and scalability of such samples, coupled with the potential for confounding factors introduced by post-mortem artefacts, presents significant challenges. In response, our adapted protocol applies this technique to stored fixed brain tissue (tissues stored for a year have been used successfully), substantially broadening its applicability and utility. We present an updated step-by-step guide for DiOlistic labelling (schematic overview Fig. 1), tailored specifically for analysing dendritic spines in fixed brain tissue, from dye preparation to data analysis. Fig. 2 illustrates a simplified example of DiOlistic labelling of a mouse hippocampus with the lipophilic carbocyanine fluorescent dyes (DiO and DiI) revealing the dendritic spines and the subsequent reconstruction of the spine structure for data analysis.

In this protocol, we outline a systematic strategy to improve the effectiveness and versatility of DiOlistic labelling for fixed tissue analysis, directly addressing the challenges and building upon the existing body of work. Our protocol is methodically organised into five distinct sections: *Tissue Preparation, Dye Bullet Preparation*, *DiOlistic Labelling*, *Confocal Imaging*, and *Image Analysis*.

2. Materials and methods

2.1. Experimental setup

2.1.1. Mice

In this protocol, we used the following mouse models:

- C57BL6/J mice, sourced from The Jackson Laboratory (strain 000664), served as wildtype controls and were aged to 1 month.
- B6.129P2(Cg)-*Cx3cr1*^{tm1Litt}/J, also sourced from The Jackson Laboratory (strain 005582), aged to 1 month for visualising the expression of green fluorescent protein (eGFP) in *Cx3cr1* $^{+}$ microglia in the brain.
- Homer1-eGFP tissue, gifted from S. Hong at the UK Dementia Research Institute (originally sourced from S. Okabe [27]), from heterozygous mice aged 7.5 months for visualising synaptic Homer1 expression.
- *App*$^{NL-G-F}$ mice, obtained from RIKEN (strain RBRC06344), aged to 6 months for facilitating amyloid plaque deposition in the brain.

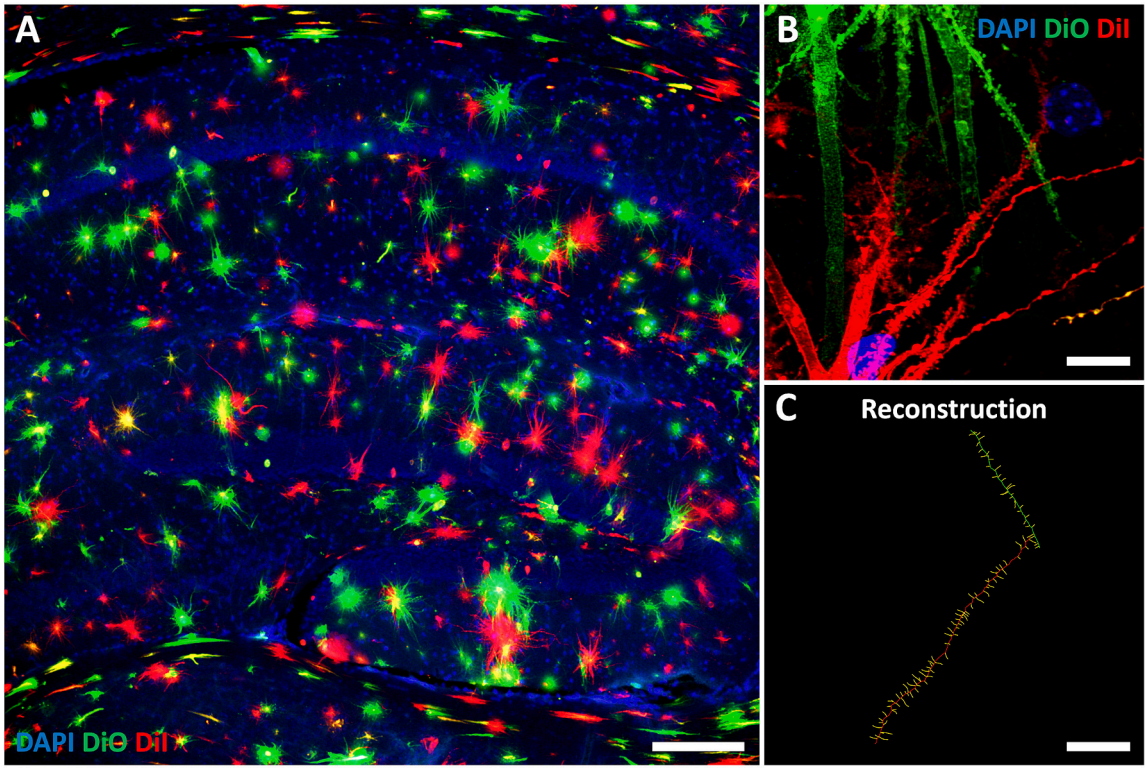

Fig. 2. Representative DiOlistic Labelling of Neurons in the Mouse Hippocampus. A) Overview of the hippocampus labelled with DiO (green), DiI (red), and DAPI (blue) for nuclei, using a 10x objective. This image showcases the random nature of the labelling. Scale bar 200 μm. **B)** Higher magnification (63x objective) view of individual dendrites and associated dendritic spines labelled in either DiO or DiI, demonstrating the detailed morphology captured by the labelling technique. Scale bar 10 μm. C**)** 3D reconstruction from confocal imaging of a neuron with DiOlistic labelling, illustrating the precise delineation of dendritic spines. Scale bar 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CRITICAL: All experiments were conducted with the approval of the Animal Welfare and Ethical Review Body (AWERB), under the oversight of the Biological Standards Committee. Experiments strictly adhered to the guidelines set forth by the UK Home Office and the Animal [Scientific Procedures] Act 1986, in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.1.2. Reagents

- Anti Iba1- 635 conjugated (FUJIFILM WAKO, Catalog No. 013–26471).
- Anti Iba1- unconjugated (FUJIFILM WAKO, Catalog No. 019–19741).
- DAPI (Thermo Fisher Scientific, Catalog No. D1306).
- Dolethal (Pentobarbital Sodium) (Vetoquinol, Catalog No. BE-V171692).

CAUTION: Pentobarbital sodium is harmful if swallowed and may cause dizziness, sleepiness, and confusion. Handle with appropriate personal protective equipment (PPE) and seek medical advice if intoxication occurs.

- DiD ‘ 1,1′-Dioctadecyl-3,3,3′,3′- Tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate’ (Thermo Fisher Scientific, Catalog No. D7757).
- DiI ‘1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perch lorate’ (Thermo Fisher Scientific, Catalog No. D282).
- DiO ‘3,3′ −Dioctadecyloxacarbocyanine Perchlorate (Thermo Fisher Scientific, Catalog No. D275).
- DPBS 1x (Gibco, Catalog No. 14190250).
- Formaldehyde 4 % for Histology, Buffered pH 6.9 (Merck, Catalog No. 1004968350).

CAUTION: Formaldehyde is toxic and carcinogenic. It may cause skin sensitisation, eye damage, and respiratory issues upon contact and inhalation. Use PPE and handle inside a fume hood.

- FluorSave Reagent (Merck, Catalog No. 345789).
- Goat anti-Rabbit Secondary Antibody, Alexa Fluor Plus 488 (Thermo Fisher Scientific, Catalog No A32731).
- Methylene Chloride (Merck, Catalog No. D7566).
- Normal Goat Serum (Abcam, Catalog No. ab7481).
- Sodium Azide (Sigma-Aldrich, Catalog No. S2002). For generation of DPBS with 0.05 % (w/v) Sodium Azide for long-term storage.

CAUTION: Sodium Azide is highly toxic and should be handled with PPE inside a fume hood. It may cause skin sensitisation, eye damage, and respiratory issues upon contact and inhalation.

- Thioflavin S (Sigma-Aldrich, Catalog No. T1892).
- Tungsten Powder, APS $<$ 1 μm, 99.95 % (Fisher Scientific, Catalog No. 11319718).

CAUTION: Tungsten powder is flammable. Keep away from heat and handle with PPE. In case of inhalation or skin exposure, clean the area and seek medical advice.

- Tween 20 (Merck, Catalog No. P1379). For generation of DPBS with 0.05 % (v/v) Tween 20 for mild permeablisation during co-labelling for immunofluorescence.

2.1.3. Equipment

- Bijou 7 ml Tubes (Fisher Scientific, Catalog No. 11369123).
- Brushes (Agar Scientific, Catalog No. AGG3443).
- Cell Culture Inserts (Scientific Laboratory Supplies, Catalog No. BR782746-24EA).
- Coverslips (Fisher Scientific, Catalog No. 11961988).
- Eppendorf Tubes- 1.5 ml (Fisher Scientific, Catalog No. 10451043).
- Glass Microscopic Slides (Fisher Scientific, Catalog No. 13192131).
- Helios Bio-Rad Gene Gun System (Bio-Rad, Catalog No. 1652432).
- Helium Gas Cylinder (BOC, Catalog No. 110745–101).
- Histology Slides (Fisher Scientific, Catalog No. 15438060).
- Leica SP8 Lightning Confocal Microscope (Leica Biosystems).
- Leica VT1200S Vibratome (Leica Biosystems).
- Multiwell plate (48 well plate) (Greiner, Catalon No. 677180).
- Parafilm (Fisher Scientific, Catalog No. 11772644).
- Pipette (Starlab, Catalog No. S7110-1000).
- Pipette Tips 1 ml (Starlab, Catalog No. S1111-6801).
- Razor Blades (Fisher Scientific, Catalog No. 11904325).
- Razor Blades Double-Edged (Fisher Scientific, Catalog No. 12043029).
- Scalpels (Swann-Morton, Catalog No. 0301).
- Slide staining tray (Simport, Catalog No. M920-2).
- Superglue (Fisher, Catalog No. 12750318).
- Tefzel Tubing (Bio-Rad, Catalog No. 1652441).

2.1.4. Software

- Data Representation: Prism 10.0.1 (GraphPad Software, Inc).
- Image/Data Analysis: Imaris Software 10.0 (Bitplane).
- Microscopy: LAS X 3.5.7.23225 (Leica).

2.2. Procedure

The protocol is divided into five distinct sections: *Tissue Preparation, Dye Bullet Preparation, DiOlistic Labelling, Confocal Imaging*, and *Image Analysis*. All timings are based on a cohort of 10 tissue samples, in this case mouse brains, for the labelling, imaging and analysis of one target brain region i.e. hippocampal CA1 apical dendritic spines.

2.2.1. Tissue Preparation (Timing ~ 7h)

Sample collection (~3h)

1) Euthanise mice of the desired model and age via an appropriate method such as an overdose of Pentobarbital Sodium.

CAUTION: Handle pentobarbital sodium carefully due to its potency as an anaesthetic and euthanasia agent.

OPTIONAL: The model choice depends on the study. For illustration of this method, we used wildtype and App$^{NL\text{-}G\text{-}F}$ mice, along with fluorescent reporter lines for microglia and synaptic puncta co-visualisation.

2) After confirmation of death, immediately perform transcardial perfusion with room-temperature 1x DPBS.

CRITICAL STEP: Avoid traditional ice-cold conditions for perfusions due to negatively impacting downstream DiOlistic labelling.

OPTIONAL: If the experimental design permits, DiOlistic labelling can be conducted on non-perfused tissue.

3) Harvest the brain, immerse in a 1.5 % formaldehyde solution for fixation in Bijou tubes and store at 4 °C for at least 72 h.

CAUTION: Formaldehyde is toxic; handle it with care in a fume hood.

CRITICAL STEP: Ensure proper fixation without additional reagents such as alcohols and permeabilisation agents. Protect specimens from light, especially if using fluorescent reporter lines.

TROUBLESHOOTING

PAUSE POINT

4) For long-term storage, switch brains to DPBS with 0.05 % sodium azide to prevent bacterial growth during storage.

CAUTION: Sodium azide is highly toxic; handle with appropriate safety measures.

5) Store the brains at 4 °C.

CRITICAL STEP: Maintain a storage temperature of 4 °C to preserve tissue integrity.

PAUSE POINT

Brain Slicing (~4h).

6) Remove the cerebellum of the harvested brain and cut 2 mm flat coronal slice from the frontal cortex with a scalpel for stable mounting.

7) Apply a drop of superglue to the vibratome stage and affix the brain posterior side up and hold until secured.

CRITICAL STEP: Ensure the brain is firmly attached to prevent movement during slicing.

8) Configure the vibratome with the following specifications: speed at 0.6 mm/s, amplitude at 1.3 mm, and section thickness at 50 μm. Fill the reservoir with DPBS and install a fresh cutting blade.

9) Proceed with slicing to produce free-floating brain sections.

CRITICAL STEP: Protect specimens from light, especially if using fluorescent reporter lines.

10) Use a fine paintbrush to gently move sections into DPBS containing 0.05 % sodium azide for preservation. Place sections in Bijou tubes or a 48-well plate, noting their order.

11) Store the sections at 4 °C to maintain tissue integrity until further use.

OPTIONAL: For immunofluorescence co-labelling, place sections in a 'staining' 48-well plate with 200 μl blocking solution (5 % normal sera in DPBS with 0.05 % (v/v) Tween 20 for mild permeabilisation for 2 h at room temperature on a rocker) for surface antigen visualisation. Aspirate and incubate with 200 μl primary antibody solution (containing 2.5 % normal sera in DPBS with 0.05 % (v/v) Tween 20 for 72 h at 4 °C on a rocker). Match the fluorescent spectrum of the target antibodies to the DiOlistic dyes, green (DiO), red (DiI) and far-red (DiD). For illustration, we used Iba1 unconjugated and fluorescent conjugated 635 antibodies. Aspirate, wash in DPBS, and transfer brain sections to clean wells. For unconjugated primary antibodies, incubate 200 μl species specific secondary antibody solution (containing DPBS with 0.05 % (v/v) Tween 20 for 4 h at room temperature on a rocker). For fluorescent conjugated primary antibodies, and sections stained with fluorescent secondary antibodies, protect from light and store in DPBS with 0.05 % sodium azide for up to 48 h until preforming DiOlistic labelling. For co-labelling with other dyes, such as Thioflavin S, stain sections with 0.1 % (w/v) Thioflavin S in DPBS for 30 min followed by DPBS washes. Plaque labelled tissue will need to be used for DiOlistic labelling within 24 h.

TROUBLESHOOTING.

2.2.2. Dye Bullet Preparation (Timing ~ 45 min)

12) Prepare two 1.5 ml microcentrifuge tubes: one for DiI dye and the other for tungsten particles.

OPTIONAL: If creating multi-dye bullets, prepare additional tubes each dye type (DiI, DiO and/or DiD) along with separate tubes for their corresponding tungsten particles. It is important to note the distinct colour emissions of each dye: DiI emits red, DiO emits green, and DiD emits far-red spectrum. This information is essential for planning for downstream analysis.

13) Weigh out 1.5 mg of DiI into its designated tube.

OPTIONAL: For multi-dye bullets, weigh out 3 mg of DiO and/or 1 mg of DiD.

14) Weigh out 40 mg of tungsten powder into the second tube.

OPTIONAL: For multi-dye bullets, weigh an additional 40 mg of tungsten for the second dye.

15) In a fume hood, dissolve DiI dye crystals by adding 500 μl of methylene chloride to its tube.

OPTIONAL: For multi-dye bullets, dissolve DiO and/or DiD separately and remain separated from DiI dye until combining at step 10.

16) Spread 40 mg of tungsten evenly on a glass slide using a razor blade to avoid clumping.

17) Add the dissolved DiI to the tungsten, 100 μl at a time, ensuring even coating.

18) Allow the methylene chloride to evaporate between additions (3 min each).

19) Once dry, agitate the tungsten particles with razor blade to ensure an even dye coating.

20) Repeat the dye addition and agitation process until all the dye has been used.

CRITICAL STEP: Add the dye solution in small increments to ensure proper coating. Adding too much solution at once without agitation may result in unusable particles.

21) After the final drying stage, agitate the particles again to prevent clumping.

CRITICAL STEP: Proper drying and agitation are necessary to prevent particle aggregation*, which can impede downstream processing.*

OPTIONAL: For multi-dye bullets, combine and agitate both dried DiI and DiO and/or DiD coated tungsten microcarriers.

TROUBLESHOOTING.

22) Seal one end of a 70 cm length of ETFE Tefzel tubing with parafilm.

23) Modify a 1 ml pipette tip to create a funnel and insert it into the tubing's open end.

CRITICAL STEP: Cut the pipette tip at the fine end and at 250 μl *line to ensure it functions efficiently as a funnel. Incorrectly cut tips can trap particles, affecting the coating process. If the tip is not cut at the* 250 μl *line, the tungsten will coat the tip rather than the tubing.*

24) Transfer the coated tungsten into the tubing using the funnel.

25) Remove the funnel and seal the tubing's end with parafilm.

26) Gently rotate the tubing to distribute the particles evenly.

CRITICAL STEP: Avoid vigorous shaking to prevent tungsten aggregation.

27) The dye-coated tungsten particles should be left in darkness on a flat surface undisturbed. This helps retain the interior coating of tungsten particles to the Tefzel tubing.

TROUBLESHOOTING.

28) Cut the tubing with a razor blade into 1.2 cm bullets. The tubing should present with a dusting of grey powder inside; this is indicative of dye-coated tungsten particles lining the tubing.

OPTIONAL: For multi-dye bullets, tubing will appear to be coated more within the tubing.

29) Store the bullets long-term in a dark place at room temperature.

CRITICAL STEP: Protect bullets from light to maintain dye fluorescence and handle them minimally to avoid dislodging the coating.

PAUSE POINT.

2.2.3. DiOlistic labelling (Timing ~ 3h)

30) Label and prepare the required histology slides on a slide staining tray, adding 1 ml of DPBS to each.

31) Retrieve the fixed brain sections from their storage.

32) Gently transfer the brain sections onto the slides using a fine paintbrush. Placing six hemisphere slices or three full coronal slices per slide and arrange the slices in a radial pattern, ensuring they do not overlap and the region of interest is centred on the slide.

CRITICAL STEP: Proper alignment is key for efficient labelling.

TROUBLESHOOTING.

33) Allow tissue sections to acclimate to room temperature for at least 10 min.

CRITICAL STEP: Labelling tissue sections directly from 4 °C storage may impair dye diffusion.

PAUSE POINT.

34) Remove excess DPBS with a pipette, taking care not to dry out the tissue.

35) Prepare the Helios Bio-Rad Gene Gun System by pressurising the helium gas to 80 PSI and perform three blank shots to stabilise pressure.

CRITICAL STEP: Initial pressurisation of the Bio-Rad gene gun does not lead to the even pressure delivery.

CAUTION: Follow *safety guidelines for operating the gene gun.*

36) Load the gene gun with prepared bullets, ensuring a tight seal.

CRITICAL STEP: Insufficient connection between the bullets and the O-ring washers impairs pressure delivery.

37) Gently blot away any remaining PBS from the slides. If necessary, reorient the slices using the paintbrush.

38) Place an inverted cell culture insert over the tissue sections.

CRITICAL STEP: Ensure sections are dry and well-positioned under the insert.

39) Fire the bullets at 80 PSI from a set distance of 1.3–1.5 cm directly above the insert. The end of the barrel should be positioned vertically and directly over the insert.

CRITICAL STEP: Maintain consistent pressure and distance for reproducible labelling.

40) Cover the tissue sections with DPBS with 1 ug/mL DAPI, being careful to maintain their orientation.

CRITICAL STEP: Labelling will primarily be on one surface, avoid sections from inverting.

41) Allow the dye to diffuse for 30 min in the dark at room temperature.

OPTIONAL: Briefly check labelling under a fluorescence microscope without drying the tissue.

TROUBLESHOOTING.

42) Fix the tissue sections by applying 4 % formaldehyde for 10 min.

CRITICAL STEP: Balance fixation to halt dye diffusion with the risk of further membrane leakage.

TROUBLESHOOTING.

43) Wash the sections three times with DPBS.

44) Dry the slides and apply FluorSave mounting media, then gently place a coverslip.

45) Apply up to four drops of FluorSave mounting media to the slides and apply a coverslip. Avoid pressing the coverslip as this could burst the labelled neurons.

CRITICAL STEP: Avoid glycerol-based media to prevent dye leakage and applying pressure to the coverslip as this will burst the labelled neurons.

TROUBLESHOOTING

46) Let the slides dry in the dark at room temperature before imaging.

2.2.4. Confocal imaging (Timing ~ 3h)

47) Prepare the Leica SP8 Lightning Confocal microscope in resonant scanning mode coupled with Lightning deconvolution for high-speed, high-resolution imaging, or alternative imaging microscope.

48) Set the imaging parameters to the following specifications for optimal image acquisition:

i. Logical size: 1024 x 1024
ii. Physical Length: 147.62 μm x 147.62 μm
iii. Pixel Sive / Voxel Size: 0.144 μm (XY) and 0.298 μm (Z)
iv. Scan mode = xyz
v. Scan Direction: Unidirectional
vi. Scan Speed: Fixed at 8,000 Hz
vii. Objective: HC PL APO CS2 63x/1.40 OIL
viii. Zoom: 1.25
ix. Pinhole: 1 AU
x. Line Average: 2
xi. Laser: OPSL 552 (emission 557–650 nm) *OPTIONAL: For DAPI use Diode 405 (emission 410–525 nm) For DiO use OPSL 488 (emission 500–550 nm), for DiD use Diode 638 (emission 650–700 nm)*
xii. Laser Intensity: 30 % – 50 %
xiii. Detector: PMT
xiv. Gain: 400 – 425

CRITICAL STEP: Keep imaging parameters consistent across sessions to ensure data uniformity.

TROUBLESHOOTING.

PAUSE POINT.

49) Mount the slide with DiOlistic-labelled tissue onto the microscope stage.

Table 1
Fresh vs fixed tissue labelling.

		Acute Fresh Tissue Labelling			Stored Fixed Tissue Labelling		
		N=1	N=10	N=20	N=1	N=10	N=20
Tissue Preparation	Tissue Harvest	15 min	2.5 h	5 h	15 min	2.5 h	5 h
	Tissue Slicing	15 min	2.5 h	5 h	15 min	2.5 h	5 h
Dye Bullet Preparation						– 45 min –	
DiOlistic Labelling	Shooting, Dye Diffusion, Tissue prep handling	1.5 h	3.5 h	8.5 h		– 1.5 h –	
	Fixation, washing and mounting		– 1.5 h –			– 1.5 h –	
Confocal Imaging (including setup)		25 min	3 h	6 h	25 min	3 h	6 h
Image Analysis		20 min	3.5 h	7 h	20 min	3.5 h	7 h
Total Active Work Time		~5 h	~18 h	~34 h	~5 h	~16 h	~27 h
Maximum single day work time		**~3.5 h**	**~10 h**	**~20 h**	**~3.25 h**	**~6 h**	**~9 h**
Scalability		Scalability for fresh tissue is limited to a maximum of 20 runs (10 twice a week) due to the necessity for concurrent *Tissue Preparation* and *DiOlistic Labelling*. In contrast, for fixed tissue, the protocol allows for *DiOlistic Labelling* and *Confocal Imaging* over consecutive days, supporting the analysis of up to 100 samples within the same timeframe.					

50) Use a lower magnification objective (10x or 20x) to identify areas of successful neuronal labelling with the Find and Mark feature.

CAUTION: Limit laser exposure to prevent bleaching the dye.

51) Switch to the 63x oil immersion objective to refine the Z-stack for each marked region, ensuring the entire depth of dendritic labelling is included.

52) Proceed to image the marked areas. Each region should span 20–40 μm in depth.

CAUTION: Maintain consistent application of immersion oil to ensure quality imaging.

53) An optimal labelling outcome should result in 10–15 imaged locations per slide, with an average turnaround time of about 10 min per mouse. Repeat for all slides.

PAUSE POINT

54) Post-imaging, apply Lightning Deconvolution using the adaptive settings for 'water-based' mounting media for best image clarity.

55) Save the original and deconvolved images in the.lif format for analysis, noting the substantial increase in file size after deconvolution.

2.2.5. Image analysis (Timing ~ 3.5 h)

56) Import the .lif files into Imaris, excluding any non-lightning images from consideration.

PAUSE POINT

57) Open the selected image for analysis and display as the orthogonal view. Activate the Filament Tracer function for dendrite and spine analysis and set the tracing parameters to 'Autopath (no loops) with Spines' for automatic dendrite and spine detection. **Supp** Fig. 1*: Original A*.

OPTIONAL: For multi-dye bullets, proceed with analysis using separate channels.

58) Focus on a Region of Interest (ROI) for detailed segmentation. Ensure that Object-Object statistics are enabled for comprehensive data analysis. **Supp** Fig. 1*: Step 1*.

59) Carefully position the ROI on the channel of interest, including adjustments in the Z-plane, to encapsulate the labelled dendrite. Avoid including extraneous dye from other dendrites or debris. **Supp** Fig. 1*: Step 1–2*.

CRITICAL: Accurate ROI placement is key for precise analysis.

TROUBLESHOOTING.

60) Begin the tracing at one end of the dendrite, adding or removing points (Shift-Left Click) as necessary without classifying seed or segment points. Refrain from selecting the starting point as a cell soma for clarity in tracing. **Supp** Fig. 1*: Step 3*.

61) Place 'Seed' points along the dendrite, again without the need for classification and generate a base dendritic shaft for the ROI. **Supp** Fig. 1*: Step 4–6*.

62) Adjust spine protrusion detection parameters as required. **Supp** Fig. 1*: Step 7–9*.

CRITICAL STEP: Consistency in spine detection settings is essential for standardised results across the dataset. Typically, either a spine head seed point of 0.45 μm and manually adjust the threshold to the bottom of the Seed point Threshold graph before the exponential increase. Or alternatively, use the default spine seed point size of 0.288 μm with automatic thresholding.

63) Review and manually correct any erroneous tracings that may have occurred, such as the software picking up adjacent dendrites or spines. **Supp** Fig. 1*: Step 10–11*.

TROUBLESHOOTING.

64) After completing the tracing of the spines, run the Classify Spines MATLAB extension to categorise spine types using user-defined parameters. **Supp** Fig. 1*: Step 12–13*.

CRITICAL STEP: Apply the following parameters systematically for uniform classification of spine subtypes based on clear presence of enlarged spine heads and spine neck:

i. Stubby: length(spine) < 1
ii. Mushroom: length(spine) < 3 and max_width(head) > mean_width(neck)
iii. Filopodia: length(spine) > 3
iv. Thin: True

65) Within the 'Statistics' tab, export the data from the 'Detailed-Average Values' section for downstream analysis. **Supp** Fig. 1*: Step 14–15*.

66) The dendrite and associated dendritic spines are reconstructed and can be saved. **Supp** Fig. 1*: Reconstruction B*.

3. Results and discussion

3.1. Development of the protocol

3.1.1. Tissue Preparation

We have recently transitioned from the conventional approach of labelling fresh brain tissue slices using DiOlistics. Our new approach addresses issues relating to tissue degeneration and, by maintaining the integrity of the tissue, the scalability of the protocol, and the potential for multiplexing the tissue labelling with other markers of cellular activity and/or pathology. As detailed in Table 1, our previous protocols for fresh tissue required up to 30 min per brain to obtain acute slices, followed by the DiOlistic labelling. Despite efforts to minimize tissue degeneration, significant post-mortem delays prior to fixation were inevitable. We therefore adapted DiOlistics to ensure that tissue was fixed as the first step in tissue processing to minimise any dendritic and synaptic changes post-mortem.

Mouse brain tissue is fixed at the point of collection using standard procedures, including PBS-Formaldehyde transcardiac perfusions or immersion fixation methods. Identifying the optimal concentration and

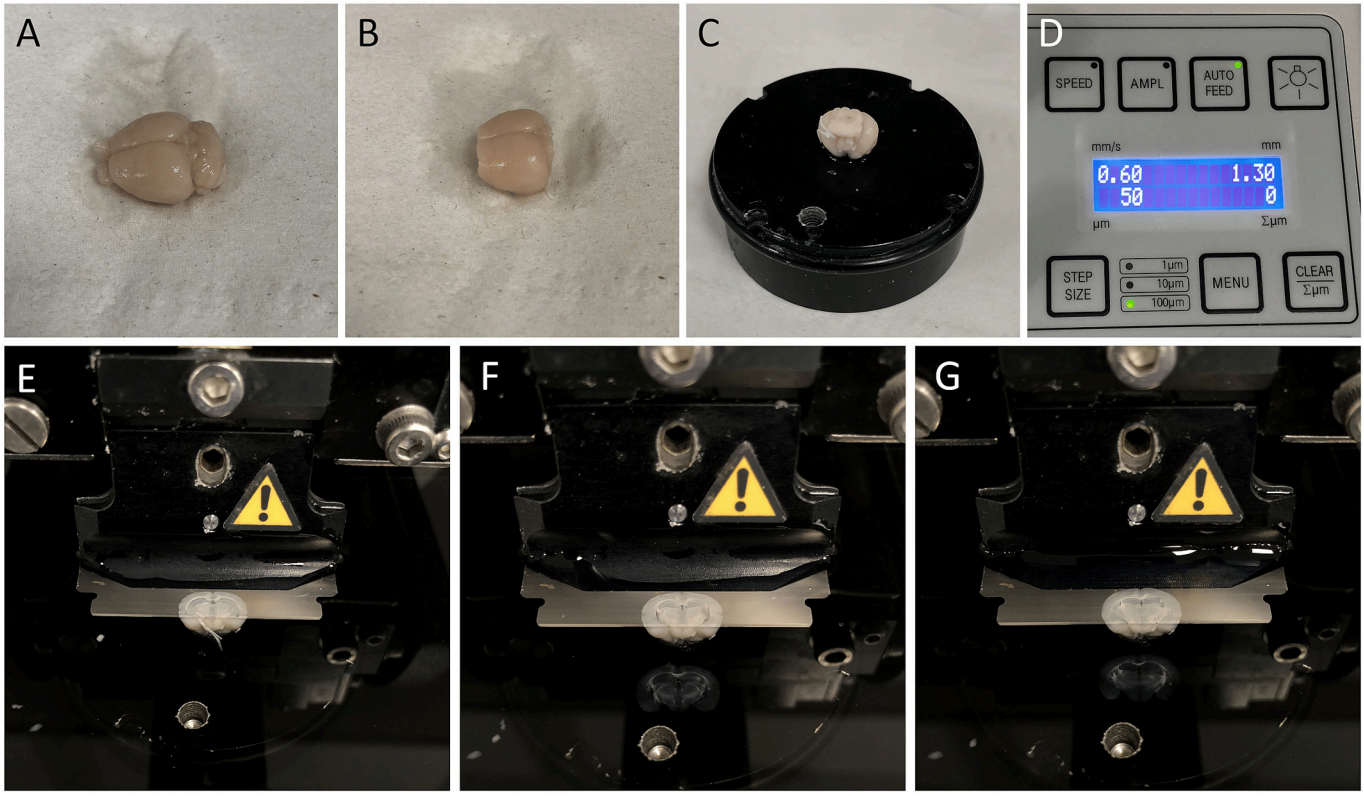

Fig. 3. Vibratome sectioning of 1.5% formaldehyde fixed mouse brain to produce free-floating tissue sections. A) Post-fixation whole mouse brain, prior to placement in the vibratome. **B)** Preparation of the brain with cerebellum removed and frontal cortex levelled for stable vibratome mounting. **C)** Brain securely mounted on the vibratome stage, posterior side up. **D)** Vibratome control panel displaying the set parameters for section thickness and speed. **E)** Initial sectioning of the brain, containing the ventral hippocampal region to demonstrate the onset of the slicing process. **F)** Mid-sectioning image, cutting optimal DiOlistic sections of the dorsal hippocampus. **G)** Collection of final hippocampal sections.

type of fixative was essential in transitioning to fixed tissue DiOlistics. Preliminary experiments indicated a 1.5 % formaldehyde solution as most effective for dye labelling which has no overt impact on the quality of DiOlistic labelling. Brain tissue stored in 1.5 % formaldehyde, or subsequently in PBS with sodium azide for long-term storage, remained viable for DiOlistic labelling for over a year, and we have experienced no difficulties in defining synaptic morphology. However, higher concentrations of fixative, such as 4 % formaldehyde had irreversible adverse impact on the quality of labelling. Transitioning to fixed tissue specimens has markedly improved the scalability and statistical robustness of our technique, allowing the inclusion of a larger cohort of individual animals in standalone experiments (Table 1), as often required for appropriate statistical analysis, and facilitating repeatable experiments.

After fixation, we recommend sectioning fixed brains with a vibratome to a thickness of up to 50 µm for free-floating sections. This thickness is ideal for producing slices that allow for detailed observation of neuronal arborisation. In our protocol, focusing on the CA1 apical dendrites of the dorsal hippocampus, a single brain typically yields up to 15 usable sections (Fig. 3). These sections can be safely stored at 4 °C in PBS with sodium azide for at least a year for labelling. Since DiOlistic labelling relies on the passive diffusion of dyes within the plasma membrane it is important to avoid steps or agents that might degrade the membrane. It is at this point, if required, that the sections can be co-labelled with additional markers and stains to complement the DiOlistic staining that is to follow with no discernible impact on synaptic detection.

Previously, fresh tissue slicing relied on rapid processing with a McIllwain Tissue Chopper (or fresh vibratome slicing), which tended to prolong the unfixed stages. To maintain tissue integrity, it's crucial to avoid exposing fixed brain sections to extreme temperatures, including freezing. Additionally, ensuring that the tissue remains in solutions free of alcohols, permeabilization agents, or sucrose is essential, as these substances can irreversibly compromise the membrane structure necessary for effective downstream DiOlistic labelling.

The adoption of this tissue preparation method for fixed brain tissue not only circumvents the challenges associated with fresh tissue processing, such as extended post-mortem delays and tissue degeneration, but also significantly enhances the efficiency and scalability of our DiOlistic labelling protocol, enabling more comprehensive and robust analyses.

3.1.1.1. Dye Bullet Preparation. DiOlistic labelling uses heavy metal particles (tungsten or gold) coated in carbocyanine dyes to label neurons. Our refined process (Fig. 4) streamlines bullet production, eliminating the tubing station and labour-intensive steps of polyvinylpyrrolidone (PVP) coating, nitrogen drying of ethylene tetrafluoroethylene (ETFE) Tefzel Tubing, and sonication for dye solution injection [10,14]. Particle preparation can be completed in as little as 45 min (Table 1). Lipophilic carbocyanine fluorescent dyes, such as DiI, are dissolved in methylene chloride and dried onto tungsten microcarrier particles. These are directly funnelled into tubing to form bullets. We have transitioned from using the original Bio-Rad's 1.7 µm diameter tungsten particles, which are no longer available, to an alternative 1 µm diameter tungsten supply (Fisher Scientific). We have noted an enhancement in downstream applications with smaller particles leading to a reduction in leakage (data not shown), presumably through smaller plasma membrane ballistic holes.

For single dye DiOlistic bullet preparation, only four specialised consumables are required: lipophilic dye, tungsten microcarriers, tubing, and methylene chloride. While combining DiI with other dyes

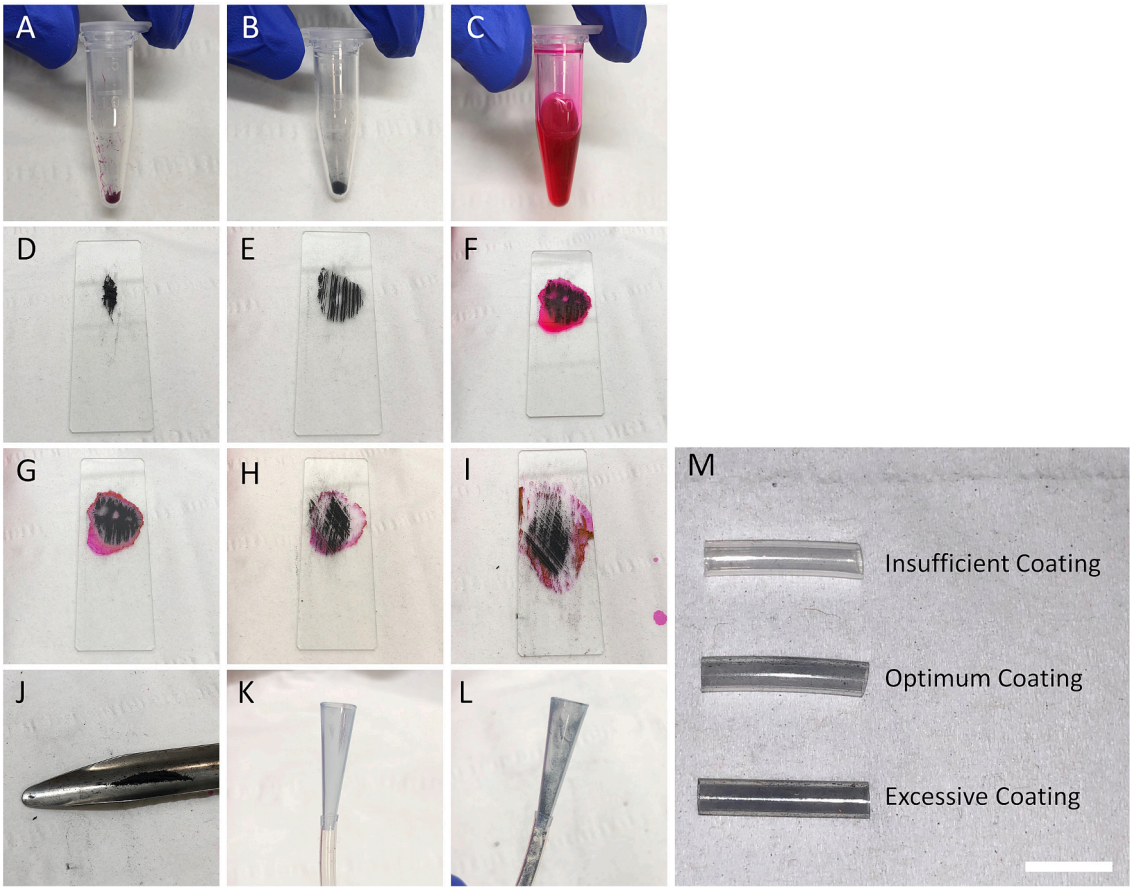

Fig. 4. Preparation of DiOlistic DiI tungsten bullets for DiOlistic labelling. A) DiI crystals. **B)** Tungsten powder. **C)** DiI dissolved in methylene chloride, creating a red solution ready for application. **D)** Tungsten powder transferred onto a glass slide in preparation for DiI application. **E)** Even distribution of tungsten powder using a razor blade on the glass slide to ensure uniform coating. **F)** Addition of DiI solution to the tungsten powder, beginning the coating process. **G)** First stage of drying, where the DiI adheres to the tungsten particles. **H)** Dried DiI-tungsten microcarriers are agitated with a razor blade. **I)** Further drying, coating and agitation of DiI to promote uniform coating of DiI onto the tungsten. **J)** DiI-coated tungsten microcarriers are transferred to surface suitable for fine pouring. **K)** A pipette tip prepared to act as a funnel for the DiI-tungsten microcarriers into the tubing. **L)** The funnel used after coating, facilitating the transfer of DiI-coated tungsten into the tubing. **M)** Comparative display of tungsten bullets, illustrating insufficient (top), optimum (centre), and excessive (bottom) DiI coating, which directly impacts labelling efficacy. Scale bar 0.6 cm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Process of Delivering DiI Bullets for DiOlistic Labelling of Mouse Brain Tissue Sections. A) Transfer of free-floating brain sections from storage to histology slides for DiOlistic labelling. Tissue is arranged in a radial pattern and equilibrated to room temperature for optimal DiI bullet decoration and diffusion. **B)** Brain sections covered with an inverted 8 μm cell culture insert to create a membrane barrier aiding the unifying the DiI bullets. **C)** Loading and firing of the Helios Bio-Rad Gene Gun with DiI bullets, prepared for delivery through the cell culture insert. **D)** Illustration highlighting the critical parameters, including the fixed distances and pressure maintained between the tissue sections and the Helios Bio-Rad Gene Gun to ensure consistent labelling. **E)** Visualisation of inverted cell culture inserts, contrasting conditions: 'new' (bottom) with no prior use, 'used' (centre) showing mild blast impact, and 'compromised' (top) indicating significant damage (red arrow) from a previous large blast impact site. Comparative display of inverted cell culture inserts, illustrating new (bottom), used (centre), and compromised with a severe previous large blast impact site. Illustration in D) created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

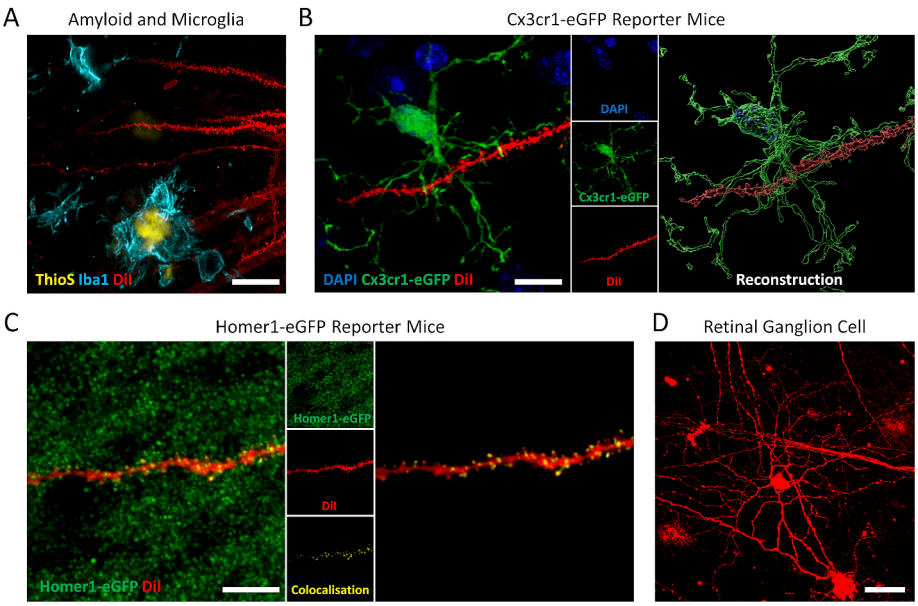

Fig. 6. Multimodal application of DiOlistic labelling. A) Co-labelling of amyloid plaque pathology (Thioflavin S, yellow) and Iba1^{+} microglia (cyan) with DiOlistics (DiI, red). Scale bar 10 μm **B)** Dual labelling in Cx3cr1-eGFP reporter mice: microglia cells (green) expressing eGFP under the Cx3cr1 promoter are visualised alongside DiI-labelled neurons (red). The inset shows the separated channels, and the right panel displays a 3D reconstruction of the microglial-neuronal interaction. Scale bar 5 μm **C)** Synaptic puncta visualisation in Homer1-eGFP reporter mice: Co-labelling (yellow) of DiI (red) with endogenous eGFP expression under the Homer1 promoter (green) reveals the distribution of synaptic puncta labelled dendrite. Scale bar 5 μm **D)** Further application of DiOlistic labelling in other fixed tissues: visualisation of retinal ganglion cells labelled with DiI (red) detailing the intricate network within the retina. Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

like DiO can increase neuronal labelling scalability, our standard practice, especially for dendritic spine analysis, is to use single-colour bullets. The pre-made bullets are cost-effective and have a prolonged shelf life; they can be stored, in the dark, at room temperature for up to 6 months.

3.1.1.2. DiOlistic labelling. We have significantly enhanced the reproducibility and practicality of this technique for fixed tissue labelling by optimising various parameters. These optimisations include fine-tuning the dye-tungsten concentration, adjusting delivery pressure relative to distance, implementing an intermediate membrane ballistic filter, determining the optimal diffusion time, and establishing the best conditions for mounting and imaging.

Under this optimized protocol for fixed tissue, the *DiOlistic Labelling* process is a separate, distinct step that follows *Tissue Preparation* (Table 1). In this phase, brain slices are transferred from storage to histology slides on a slide tray. Up to six hemisphere slices or three full coronal slices are arranged centrally in a petal formation on each slide and dried with a tissue (Fig. 5). Slices are covered with an inverted 8 μm pore size cell culture insert, and subjected to DiOlistic labelling at 80 PSI using a Helios Bio-Rad Gene Gun loaded with optimised dye-tungsten bullets fired through the original accelerator chamber without the spacer or the O'Brien *et al.* modified barrel [10] (Fig. 5).

After the DiOlistic labelling process, the tissue slices require at least 30 min at room temperature to allow for adequate dye diffusion along the dendrites. The diffusion period can be synchronised with the nuclei staining process in DPBS. Once the dye has thoroughly diffused and labelled the neurons, the slices are fixed in a 4 % formaldehyde solution to halt further dye migration. They are then mounted in glycerol-free mounting media under a coverslip, ready for visualisation and imaging. In contrast to the fresh tissue process, the discrete *DiOlistic Labelling* phase eliminates the time-consuming monitoring needed during fresh tissue dissection, incubation, and handling. Our protocol supports a production line approach to tissue handling, allowing sequential labelling of neurons in large cohorts of samples, typically up to 20 mice at a time, equating to 200 – 300 images of labelled cells per session (Table 1).

3.1.1.3. Confocal imaging. The use of a confocal microscope is essential for visualising DiOlistic labelling in brain slices, enabling the three dimensional resolution of individual dendritic spines. We have also optimised the imaging procedure to obtain high-quality images for dendritic spine analysis from a single mouse, targeting one brain region, requiring just 10–15 min per mouse. This enhancement significantly increased the scalability of *Confocal Imaging* (Table 1). There are many suitable confocal imaging setups capable to capturing the DiOlistic labelled neurons, we use a Leica SP8 confocal imaging system, equipped with Resonant scanning technology. This advanced feature facilitates the rapid acquisition of high-resolution 3D images of the labelled dendrites. Following image capture, Lightning Deconvolution is applied to further refine the visualisation of individual dendritic spines. The system's 'Mark & Find' feature, combined with the use of separate Z stacks, adds an element of automation to our process. This enables us to achieve individual image turnaround times for dye labelling in as little as 30 sec, all while capturing the detailed 3D structure of the dendrites.

It is critical to note that neurons with DiOlistic labelling from fixed brain tissue slices must be imaged within six hours to prevent artefacts related to leaky membranes caused by prior fixation. Therefore, an efficient imaging schedule is imperative. Balancing the number of samples labelled with the available imaging resources within the same day is crucial to ensure optimal imaging results.

3.1.1.4. Image analysis. Optimising downstream analysis pipelines is essential for managing the large imaging datasets generated from the DiOlistic labelling procedure. Various software options are capable of effectively analysing DiOlistic-labelled dendritic spines, including free software. However, we primarily use Imaris (Bitplane) and its integrated Filament Tracer module, in conjunction with the MATLAB spine classifier function. This combination facilitates precise and efficient analysis of these complex datasets. The user's familiarity with the software plays a role in the analysis speed (Table 1).

The tracing process of dendritic spines involves several core steps (**Supp** Fig. 1): The image analysis process is semi-automated, starting with a user defining regions of interests. The dendritic shaft and dendritic spine protrusions are then automatically traced within this ROI. The tracing is then manually checked for accuracy and requires minimal user intervention. The spines are then categorised based on predetermined morphological parameters, with the data exported for further analysis. A semi-automated approach strikes a balance between accuracy and high throughput.

3.2. Applications of the method

DiOlistics removes the need for costly interbreeding with transgenic reporter mouse lines, such as Thy1-GFP, for spine visualisation, making it accessible for already established mouse lines. We have refined and streamlined the stages of the protocol to enhance the efficiency of the DiOlistic labelling process.

Incorporating DiOlistic labelling with fixed tissue facilitates the simultaneous application of additional dyes and the detection of cell-surface antigens via immunofluorescence. For instance, as demonstrated in Fig. 6, DiOlistic labelling can be adeptly paired with prior labelling of amyloid plaque markers such as Thioflavin S and concurrently stained with antibodies, such as to identify proximate microglial cells using Iba1 (Fig. 6 **A**). It is crucial to note, however, that conventional immunofluorescence protocols involving permeabilisation agents and alcohol can irreversibly alter membrane integrity, which is detrimental to the efficacy of DiOlistic labelling. Furthermore, DiOlistic labelling can be effectively combined with other reporter mouse lines. We have successfully integrated DiOlistic labelling with reporter lines for microglia (Cx3cr1-eGFP) and synapses (Homer1-eGFP) (Fig. 6 **B-C**). This demonstrates the method's versatility and potential for broader applications.

While our protocol primarily focuses on labelling dendritic spines within the hippocampus, its unbiased and random nature makes it highly adaptable for other neuronal subpopulations throughout the brain and in other tissues such as the retina [25] (Fig. 6 **D**). The inherent randomness of DiOlistic labelling means it can effectively target neurons in multiple brain regions, extending the technique's potential use beyond the hippocampus. Adaptability to various brain target regions is dependent on the neuronal density of the region of interest, the protocol can be modified as necessary, with suggestions available in our troubleshooting section. Adjustments such as increasing the dye concentration, modifying the dye delivery pressure, and increasing dye diffusion time (e.g. 100 PSI firing pressure and dye diffusion time up to 24 h for retinal tissue) can be made to suit specific regional requirements.

The higher sample throughput enables larger experiments with appropriate statistical power and logistical capacity for broader questions, e.g. including sex as a parameter. Additionally, the fixation and sample storage permit re-labelling of, and/or repurposing of, the remaining tissue, which not only allows more valuable interrogation of samples, but reduces the number of animals used in such studies.

3.3. Limitations

While DiOlistic labelling is a powerful tool for studying dendritic spines, it is important to recognise some inherent limitations that arise from its physical properties and labelling process, particularly during tissue collection.

One such limitation is that neuronal membranes can be irreversibly compromised by exposure to 'normal' histological procedures, such as membrane permeabilisation typically used in other techniques [26]. This compromise can affect the integrity of the labelled structures and potentially impact the subsequent analysis. Such limitations notably arise when tissue is subjected to certain conditions during preparation. For instance, fixing tissue in formaldehyde concentrations higher than 1.5–2 % or using alternative fixatives can lead to ineffective labelling. Similarly, exposing tissue to extreme temperatures, either below 4 °C or during incubations at elevated temperatures like 37 °C, can compromise the efficacy of the labelling process. Tissue specimens should be stored at 4 °C and it is imperative to allow them to return to room temperature prior to undergoing DiOlistic labelling. Additionally, contact with certain reagents, such as alcohols or permeabilisation agents, can adversely affect tissue integrity. Therefore, meticulous attention to tissue handling is crucial to mitigate these limitations. For example, because of these constraints, this technique is not suitable for cryosections.

In DiOlistic labelling, achieving an optimal labelling intensity is crucial to ensure high quality neuron visualisation in the region of interest. Ideally, the technique should result in a random, sparse decoration of labelled neurons. Under labelling, while less concerning, can still be addressed effectively. If it occurs due to technical issues during delivery, tissue sections can be re-labelled using the same bullets or an alternative batch. However, over labelling poses a more significant challenge, as it can obscure individual neuron identification. Over labelling typically occurs due to excessive dye concentration or density in the tissue section, leading to increased dye deposition and consequent membrane leakage. In such instances, it is advisable to repeat the labelling on additional tissue sections.

In the context of vibratome tissue sections for DiOlistic labelling, we recommend using free-floating sections that are greater than 35 μm in thickness, with an ideal thickness of approximately 50 μm. This recommendation is based on two primary considerations. Firstly, this thickness allows for sufficient three-dimensional space for effective labelling. Secondly, the DiOlistic labelling process typically achieves a physical depth of around 50 μm, which aligns with the limitations of confocal microscopy's optical properties. It is also important to note that using sections thicker than recommended may lead to additional artefacts, such as compression issues when mounting the tissue under a coverslip.

The optimisation of dye delivery pressure is crucial in DiOlistic labelling. The pressure is essential not only for enabling the particles to penetrate individual neurons but also for providing enough energy for dye diffusion along membranes. However, applying high pressure can lead to substantial impact holes on fixed membranes, creating sites for dye leakage and potentially causing neuronal membranes to burst. To mitigate this issue, the protocol incorporates an inverted 8 μm pore size cell culture insert. This setup allows us to maintain a delivery pressure of 80 PSI. The insert serves as a barrier, obstructing large aggregated particles, and thereby fine-tuning the dye delivery onto the tissue sections. It is important to note that pressures below 80 PSI are often inadequate for allowing sufficient dye microparticles to penetrate through the insert. Although we have experimented with inserts of various pore sizes, consistent replication and optimal results have been achieved using the 8 μm pore size inserts.

Another critical aspect to consider is the process of mounting the tissue sections. Care must be taken not to apply direct force onto the coverslip, as this can cause the dye to burst from neuronal membranes, compromising the labelling. Therefore, slides must be handled with utmost care once labelled. We have found that using glycerol-free mounting media during the coverslip mounting of dye-labelled tissue sections significantly improves dye retention in the membranes. However, it's important to recognise that despite the efficiency of our labelling process in rapidly delineating neuronal morphology, dye retention in fixed brain tissue is inherently limited. Other derivates of DiI, such as CM-DiI, may offer longer dye retention which might be beneficial in some circumstances, but is not cost effective [15]. As a result, to ensure optimal visualisation, labelling and imaging should ideally be conducted on the same day, since labelled neurons may not retain the dye effectively over multiple days.

The confocal imaging of labelled neurons necessitates a largely manual process due to the requirement for high-resolution z-stack images from sparsely labelled locations. While automated scanning of

Table 2
Troubleshooting table.

Steps	Problem	Possible reason	Solution
3, 6-11	Poor quality of tissue slices	Suboptimal tissue harvest and fixation	Ensure proper fixation by using the correct 1.5 % formaldehyde concentration. Incorrect fixation can affect the integrity of the tissue, leading to poor slicing quality. Exercise caution during tissue dissection to avoid damaging the tissue. If you notice separation (in the mouse brain this is commonly in the cortex), consider reducing the vibratome blade speed to 0.4 – 0.5 mm/s and amplitude to 1 mm for smoother sectioning
		Inadequate positioning of the tissue or blade on the vibratome	Carefully position the tissue centrally on the vibratome base and secure it on a flat surface. Check the blade alignment to ensure it's not at an angle. Correct positioning is crucial for even slicing
18-22	Uneven dye coating of tungsten particles, resulting in large clumps	Insufficient drying time for methylene chloride	Allow at least 3 min for methylene chloride to evaporate after each addition of the dye solution. Increase drying time if necessary. If the tungsten-dye particles coat the razor blade, clean the blade with an additional razor to ensure proper agitation
21, 26-27	Dye-coated tungsten particles do not adhere inside the tubing and fall out	Inadequate agitation of the dye-coated tungsten, leading to clumping	Increase drying and agitation time for dye-coated tungsten on the glass slide before funnelling into the tubing. Ensure thorough dispersion of particles to avoid clumping
		Excessive rotation of the tubing during filling	Rotate the tubing gently and vertically to evenly distribute the particles along the interior. Avoid over-rotating to prevent dislodging the particles. If the particles do not adhere to the tubing, allow additional resting time
13-22, 32-41	Inadequate or excessive DiOlistic labelling	Incorrect dye or tungsten quantity in bullets	Verify that the dye, tungsten, and methylene chloride are measured accurately as per the DiOlistic Preparation guidelines. Precise quantities are crucial for optimal labelling. For multicolour bullets, ensure to keep different dyes separated until the final drying step to prevent cross colouring.
		Slices not positioned correctly or reached room temperature under the blast radius	Align the tissue slices in the centre of the slide and ensure they are within the blast radius of the Helios Bio-Rad Gene Gun. Avoid overlapping slices as it impedes labelling and ensure tissue slices have neutralised to room temperature to aid in dye diffusion.
		Presence of PBS layer over tissue sections	Dry the tissue sections to remove any residual PBS. Any PBS layer can act as a barrier during particle delivery
		Inappropriate pressure settings and positioning of the Gene Gun	Confirm that the Helios Bio-Rad Gene Gun is set to the correct pressure and is at the correct distance from the tissue sections. Check the installation and fit of the bullets in the cartridge holder and the barrel's alignment with the output pressure
		Dye diffusion time	Increase dye diffusion time to allow for more dye to fill the neurons, taking consideration that additional diffusion in fixed tissue may lead to an increase in dye leakage
		Damaged or punctured cell culture insert	Regularly inspect the cell culture insert for damage. Replace the insert if the membrane is compromised to ensure proper labelling
3-5, 42, 45	DiOlistic labelling appears leaky	Compromised tissue integrity due to fixation or storage conditions	Use the correct 1.5 % formaldehyde fixation and avoid storing tissue under 4 °C or exposing it to heat. Ensure no contact with alcohols or permeabilisation agents that can affect membrane stability. Consider post labelling 4 % formaldehyde fixation is sufficiently washed and from the tissue sections
		Compression of tissue during handling	Handle tissue slices and coverslips carefully to prevent compression. Any force exerted can cause the dye to burst from the cell membranes, leading to leakage
40-45	DiOlistic labelling appears blurry and out of focus	Tissue sections may have flipped over during the mounting process	Take extra care during the washing and mounting steps to prevent the tissue sections from flipping over on the slide. This can be achieved by gently handling the slides and using tools like fine paintbrushes for adjustments. Ensure that the labelled side of the tissue remains facing up throughout the process, especially when removing liquids or applying mounting media
48, 58-59, 63	Difficulty in tracing dendritic spines in Imaris	Suboptimal image quality for tracing	Ensure that the images captured have sufficient dye intensity for effective tracing. If the brightness or clarity of the dendrites is low, Imaris may struggle to trace accurately. Reassess the confocal imaging parameters to improve dye intensity and overall image quality
		Incorrect positioning of the region of interest including extraneous dye or debris	When setting the region of interest for tracing, be cautious of including nearby dendrites or debris, particularly if they are brighter than the target dendrite. Imaris uses local thresholding within the selected area; thus, extraneous bright objects can disrupt the tracing process. Adjust the region of interest to focus solely on the target dendrite and exclude adjacent structures or debris

slides would be ideal, it is currently unfeasible due to the resolution demands (sub 0.5 μm interval) for visualising dendritic spines, the extensive Z stack requirements, and the sparse nature of the labelling. Furthermore, the imaging process must be conducted quickly. Unlike fresh tissue samples where dye labelling is relatively resistant to prolonged confocal imaging, fixed tissue labelled neurons contain less dye and are more prone to photobleaching. Therefore, extended imaging periods are not advisable for fixed tissues.

Although we utilise Imaris and its Filament Tracer module for rapid data acquisition, manual input remains a crucial aspect of the analysis process. This is primarily due to the necessity of accurately positioning regions of interest to guide the software's tracing, which is essential given the random nature of dye labelling. Current software limitations pose challenges in tracing larger areas and effectively excluding artefacts caused by dye debris. Additionally, manual intervention is required to correct misrenderings where the software may incorrectly interpret labelling, such as mistaking adjacent spines from nearby dendrites. This underscores the importance of careful manual oversight throughout the image analysis process.

While DiOlistic labelling presents certain inherent challenges, such as the precise handling of tissue samples and the intricacies of dye delivery and imaging, these limitations do not detract from its overall utility as a powerful tool in neuroscientific research. Each constraint highlights an area for potential refinement, signalling opportunities for future technological and methodological advancements. We anticipate possible further improvements in accuracy, efficiency, and adaptability, further solidifying DiOlistic labelling's role in advancing our understanding of neuronal structures and functions.

3.4. Conclusion

In summary, we have refined and developed an optimised DiOlistic labelling protocol for fixed tissue, enhancing the visualisation of dendritic spines in stored tissue specimens and facilitating the integration of multiplex histochemical detections. The protocol's comprehensive approach, from tissue preparation through to confocal imaging, ensures high-quality, reproducible results. It offers broad applicability across various neuronal populations and enables detailed morphological studies of neurons *in situ*. By streamlining the DiOlistic labelling process, this work significantly advances the technique capabilities, allowing for a deeper understanding of the structural basis of neuronal function and connectivity.

Troubleshooting

Troubleshooting advice can be found in Table 2.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Author contributions.

R.J.B. and P.R.T. conceived the project and developments to the protocol. R.J.B. performed all stages of the DiOlistic labelling protocol related to the brain. G.C. performed DiOlistics on fixed retina and assisted with protocol optimisation. J.E.M established DiOlistic labelling setup in the retina. R.J.B. and P.R.T wrote the manuscript with inputs from all authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2024.06.009.

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