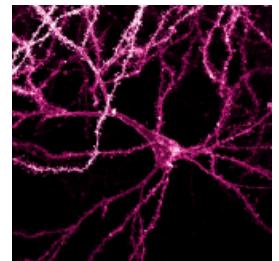


Aug 13, 2024

## Dil Labelling with a Paintbrush: a Low-cost Alternative to DiOlistic Labelling in Neurons

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A low-cost method for hig...



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**We use this protocol and it's working**

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## Abstract

DiOlistic labelling is a high-throughput technique commonly used to label neurons in fixed brain tissue using the fluorescent lipophilic carbocyanine dye, Dil. It labels a large population of neurons in a dense yet distributed pattern, making it ideal for morphological studies. However, DiOlistic labelling typically requires an expensive commercial gene gun. Therefore, our protocol presents a low-cost alternative using materials that are already available in most laboratories (e.g. a plastic 12-well culture plate lid) or easily acquired at a low price (e.g. a paintbrush). We detail the Dil labelling process, including the preparation, delivery, incubation, and post-processing steps. Overall, this protocol labels a large population of neurons in a dense yet distributed pattern and, therefore, is a simple and low-cost alternative to DiOlistic labelling.

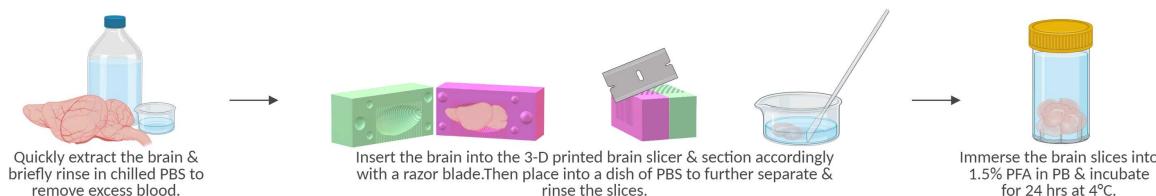
## Guidelines

The **step-by-step protocol** only describes the **Dil labelling** process. This **guidelines section** provides **reference information** regarding the **other procedures**.

**A typical workflow of the procedures involved:** Tissue Preparation (Sectioning & Fixation) → Dil Labelling (Dil Preparation, Dil Delivery, Dil Incubation, & Dil Post-processing) → Tissue Mounting → Image Acquisition → Post-processing (e.g. deconvolution) → Morphological Reconstruction

### Tissue Preparation

- This protocol used fresh, fixed adult rat brain tissue that was prepared by rapidly slicing into 800 µm coronal sections using a **custom 3D printed brain slicer** and fixed by immersing into 1.5% PFA in PB for 24 h at 4°C. The tissue was then stored in PBS at 4°C until Dil labelling (see the figure below).
- Various tissue preparation methods and sample types are compatible with this method. Depending on the sample type, fixation can be done with intracardial perfusion or by immersion fixation, but generally, lower aldehyde concentrations (1.5% or 2% PFA) are preferred over the traditional 4% PFA as higher concentrations impede Dil diffusion and the quality of labelling (although many protocols still use 4% PFA) (Cheng et al., 2014; Kim et al., 2007; Staffend & Miesel, 2011).
- Most protocols use a vibratome to section the tissue, and most commonly use sections with a thickness of around 200-400 µm. The tissue sections tend to be thicker than traditional IHC methods to ensure the sampling of complete neurons/minimise truncations. Cryostats and freezing microtomes are avoided, as freezing will disrupt the cell membrane. Successful labelling impinges on an intact membrane (Gan et al., 2009).
- Methods for fixed samples: rat (Rasia-Filho et al., 2010), rats & mice (Foster-Olive et al., 2018), non-human primate & mice (Seabold et al., 2010), human (Das et al., 2019), and neuronal cultures (Cheng et al., 2014; Kashiwagi et al., 2019).
- Unfixed samples can also be used (although less commonly). However, they require much shorter Dil incubation times, alternative post-processing procedures, and, of course, a shorter visualisation limit.
- Methods for unfixed samples: fish & mice (O'Brien & Unwin, 2006), rat (acute slice preparation) (Hosokawa et al., 1992), & unfixed cultures (Honig & Hume, 1986).
- It is crucial to note that the tissue's surrounding environment, especially during sectioning and fixation, can cause extraneous changes to the dendritic morphology. See the following literature (Bourne et al., 2007; Dehghani et al., 2018; Fiala et al., 2003; Idziak et al., 2023; Kim et al., 2007; Kirov et al., 2004; Korogod et al., 2015; Park et al., 1996; Tao-Cheng et al., 2007).
- Try to control for these factors as much as possible. However, if not possible, at least ensure the preparation conditions are systematic and consistent amongst experimental samples and be aware that these artifacts can arise.



*Schematic of the tissue preparation of fresh fixed coronal sections (800 µm) from adult rat brains.*

## Dil Labelling

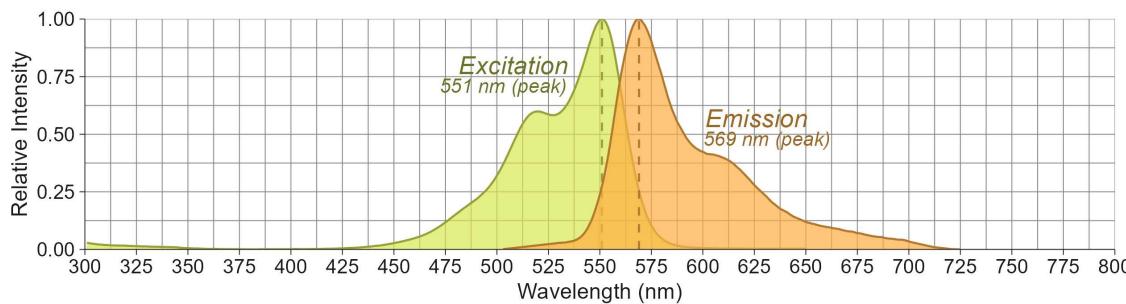
We strongly recommend reviewing the provided notes and video and conducting a pilot study or at least working with test samples before proceeding to experimental samples.

*Dil labelling can be combined with other procedures, such as:*

**Multi-colour labelling** - Multi-colour labelling can be achieved by combining combinations of Dil, DiO, and Dil as in the original DiOlistic labelling study (see Gan et al., 2000; Gan et al., 2009).

**Immunofluorescence** - Dil labelling can be coupled with immunofluorescence techniques; however, some modifications may be needed. Particular care should be taken regarding the permeabilization reagent and tissue thickness (see Matsubayashi et al., 2008; Neely et al., 2009; Staffend & Miesel, 2011).

**Tissue Clearing** - Tissue clearing may be helpful for thick optical sections. Many tissue clearing methods are incompatible with lipophilic dyes, like Dil, mainly due to the delipidation steps. However, there are Dil-compatible methods available (see reviews by Richardson & Lichtman, 2015; Tian et al., 2021).



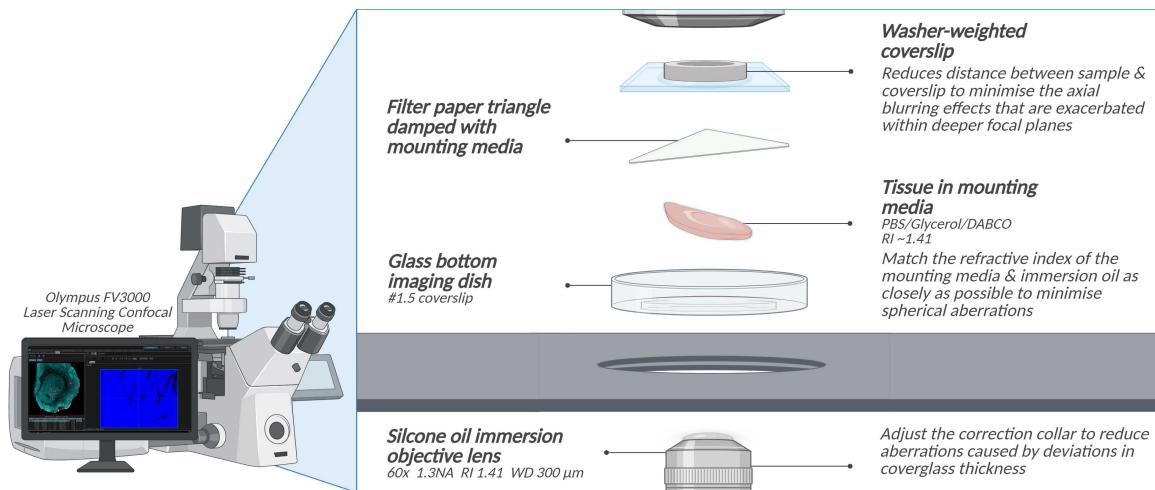
*Spectral data of Dil (dye). Data obtained from fpbase.org. Use [FPbase](#) &/or [SpectraViewer](#) to determine compatibility with other fluorescent markers & dyes.*

## Tissue Mounting

- For our 800 µm thick brain tissue slices, we developed a temporary mounting method allowing imaging of both sides. We used a glass-bottom imaging dish (ibidi µ-Dish 35 mm, high, #1.5) with a washer-weighted coverslip to minimize the distance between the sample and the coverslip. This setup allowed easy flipping of the tissue for imaging of both sides (see figure below).
- We used a mounting media containing a mix of ½ glycerol and ½ PBS with 2.5% DABCO antifade (refractive index (RI) ~1.41, matching the RI of the silicone immersion oil of the objective lens). The mounting media also had a mild clearing effect due to its similarity to fixed brain tissue's RI.
- However, the Dil-labelled tissue was not left in the mounting media for extended periods as glycerol has a dye-bleeding effect on membrane dyes like Dil (Cheng et al., 2014; Murphy & Fox, 2007). Still, many protocols use glycerol-based mounting media (Gan et al., 2009; Kim et al., 2007; Staffend & Miesel, 2011) or commercial glycerol containing mounting medias such as Prolong Gold (Cheng et al., 2014; Seabold et al., 2010) or Vectashield (Trivino-Paredes et al., 2019).
- Also note that some mounting media, particularly hard-setting ones, can induce tissue shrinkage and compression, potentially altering the neuronal morphology (see interesting [discussion thread](#)).
- Traditional mounting and coverslipping can be used for thinner tissue sections featured in other protocols (sometimes with a spacer in between to avoid crushing the tissue) (Foster-Olive et al., 2018; Heck et al., 2012; Rasila-Filho et al., 2019).

2010).

- However, it is crucial to note optimisation of the mounting parameters is paramount to successful imaging, especially when it comes to imaging small structures like dendritic spines (see Banecelin et al., 2021; Dumitriu et al., 2011; Foster Olive et al., 2018; Kashiwagi et al., 2019; Peterson et al., 2015). As such, it is essential to minimise the effect of spherical aberrations as much as possible (Diel et al., 2020; Murray, 2011)!



*Schematic of tissue mounting setup for thick sections (800  $\mu\text{m}$ ).*

## ***Image Acquisition, Post-processing, & Morphological Reconstruction***

*These topics are beyond the scope of this protocol, however, some relevant resources & literature are listed below.*

### ***Neuronal Reconstruction***

- (Allen Cell Types Database, 2018) Technical White Paper: Cell Morphology And Histology (v.7)
- (Allen Institute for Brain Science, 2021) 3D Reconstruction of Neurons in Vaa3D V.3
- (Arshadi et al., 2021) SNT: A unifying toolbox for quantification of neuronal anatomy
- ImageJ SNT <https://imagej.net/plugins/snt>
- NeuroMorpho.Org <https://neuromorpho.org>
- (Parekh & Ascoli, 2013) Neuronal Morphology Goes Digital: A Research Hub for Cellular and System Neuroscience
- (Wu et al., 2004) High-throughput Morphometric Analysis of Individual Neurons

### ***Dendritic Spine Morphology***

- (Das et al., 2019) Dil-mediated analysis of presynaptic and postsynaptic structures in human postmortem brain tissue
- (Dumitriu et al., 2011) High-throughput, detailed, cell-specific neuroanatomy of dendritic spines using microinjection and confocal microscopy
- (Foster Olive et al., 2018) Diolistic Labeling and Analysis of Dendritic Spines
- Harris Lab Wiki <http://tinyurl.com/harrislabwiki>
- (Heck et al., 2012) A deconvolution method to improve automated 3D-analysis of dendritic spines: application to a mouse model of Huntington's disease
- (Kashiwagi et al., 2019) Computational geometry analysis of dendritic spines by structured illumination microscopy

- (Ofer et al., 2022) Structural Analysis of Human and Mouse Dendritic Spines Reveals a Morphological Continuum and Differences across Ages and Species
- (Okabe, 2020) Recent advances in computational methods for measurement of dendritic spines imaged by light microscopy
- (Paternoster et al., 2018) The importance of data structure in statistical analysis of dendritic spine morphology
- (Pchitskaya & Bezprozvanny, 2020) Dendritic Spines Shape Analysis—Classification or Clusterization?
- (Zaccard et al., 2021) Protocol for live enhanced resolution confocal imaging of dendritic spinule dynamics in primary mouse cortical neuron culture

### **Both &/or General Neuroscience**

- Allen Brain Map - Morphological Annotation <https://portal.brain-map.org/explore/connectivity/synaptic-physiology/synaptic-physiology-experiment-methods/cell-classification#morphology>
- (Dickstein et al., 2016) Automatic Dendritic Spine Quantification from Confocal Data with Neurolucida 360
- EBRAINS Tools <https://www.ebrains.eu/tools>
- (Harris & Spacek, 2016) Dendrite Structure
- (MBF Bioscience, 2015) Webinar: Neuronal imaging protocols/techniques for 3D neuron reconstruction [https://youtu.be/HczuQjeNcR4?si=GKEHj\\_Nrnl3jZ7Xi](https://youtu.be/HczuQjeNcR4?si=GKEHj_Nrnl3jZ7Xi)
- Mouse Brain Atlas <https://labs.gaidi.ca/mouse-brain-atlas>
- Neuroimaging Informatics Tools and Resources <https://www.nitrc.org>
- Rat Brain Atlas <https://labs.gaidi.ca/rat-brain-atlas>
- SynapseWeb <https://synapseweb.clm.utexas.edu>

### **General Imaging & Post-Processing**

- Artifacts & Aberrations in Deconvolution Analysis <https://www.olympus-lifescience.com/en/microscope-resource/primer/digitalimaging/deconvolution/deconartifacts>
- (Bankhead, 2014) Analyzing fluorescence microscopy images with ImageJ
- ImageJ NeuroCyto LUTs <https://sites.imagej.net/NeuroCyto-LUTs/luts>
- (Jacquemet et al., 2020) The cell biologist's guide to super-resolution microscopy
- (Jonkman et al., 2020) Tutorial: guidance for quantitative confocal microscopy
- (Jost & Waters, 2019) Designing a rigorous microscopy experiment: Validating methods and avoiding bias. (*see the additional resources listed in table 1*)
- (North, 2006) Seeing is believing? A beginners' guide to practical pitfalls in image acquisition
- OpenOcular OE2 Adapter <https://www.openocular.com>
- (Pawley, 2000) The 39 Steps: A Cautionary Tale of Quantitative 3-D Fluorescence Microscopy
- (Schermelleh et al., 2010) A guide to super-resolution fluorescence microscopy
- (Shaw & Rawlins, 1991) The point-spread function of a confocal microscope: its measurement and use in deconvolution of 3-D data
- SVI Microscopy Nyquist rate and PSF calculator <https://svi.nl/Nyquist-Calculator>
- (Wallace et al., 2001) A Workingperson's Guide to Deconvolution in Light Microscopy

## Materials

### Reagents

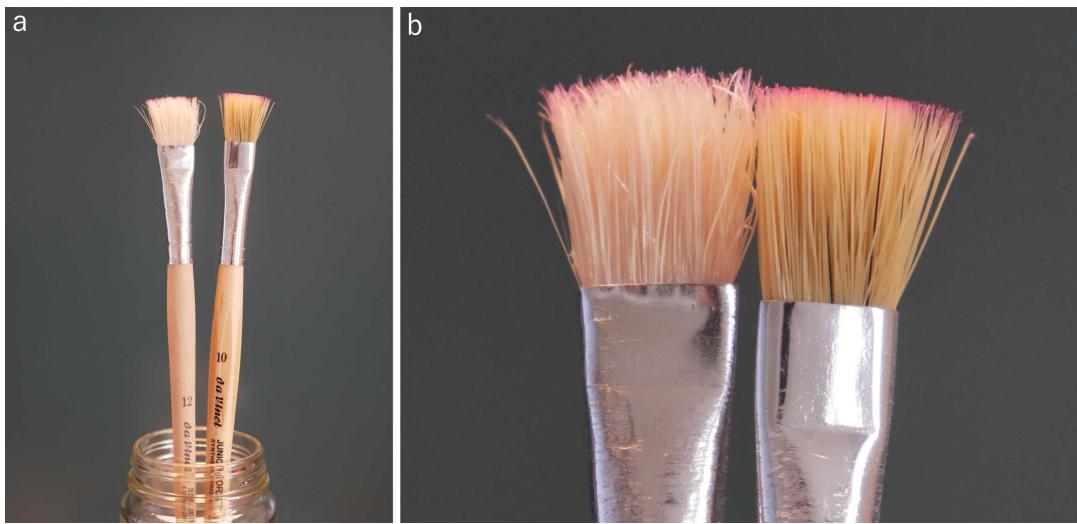
1.  100% Ethanol **Contributed by users**
2. DAPI in PBS (1:50000)  DAPI Staining Solution **Abcam Catalog #ab228549**
3.  Dil **Abcam Catalog #ab145311**
4. 4% paraformaldehyde (PFA) in 10 mM PBS (pH 7.4)
5. 10 mM phosphate-buffered saline (PBS) (pH 7.4)

### Consumables

1. Aluminium foil
2. Amber glass reagent bottle
3. ddH<sub>2</sub>O or Milli-Q water (optional)
4. Falcon tube (50 mL conical centrifuge tubes, Falcon)
5. Kimwipes (Delicate task wipers, Kimtech Science)
6. Plastic 12-well cell culture plate with lid (see details below)
7. Silica desiccant packet (optional)
8. Small container (28 mL flat bottom container, Techno Plas)
9. Ziplock bag or airtight container (optional)

### Equipment

1. Analytical scale
2. Fumehood
3. Glass bottom imaging dish ( $\mu$ -Dish 35 mm, high Glass Bottom, #1.5, ibidi) (optional)
4. Glass plate/dish (~10 cm diameter) (optional)
5. Metal forceps
6. Paintbrush for tissue slice transfer (Water brush flat tip, Tombow)
7. Paintbrush for Dil delivery (Student Bristle Flat, **series 29 no. 12**, da Vinci)
8. Paintbrush for Dil delivery (Junior Borste Synthetic Bristle Brush Flat, **series 329 no. 10**, da Vinci)
9. Pipette
10. Refrigerator
11. Vortex



*Paintbrushes for Dil Delivery.* **a)** A side-by-side comparison of the paintbrushes. **b)** A close-up view of the bristles. The **series 329 no. 10 brush** (on the right) has synthetic bristles that are more densely packed with smaller bristle tips. This brush produces a denser labelling pattern with smaller crystals. Whereas the **series 29 no. 12 brush** (on the left) has natural bristles that are less densely packed & tend to produce a lower density of labelling with larger Dil crystals. Paintbrushes with similar characteristics will likely work, too (e.g. flat/bright-shaped paintbrushes with bristles for oil or oil/acrylic paints).

## Equipment

### CELLLSTAR 12 Well Cell Culture Multiwell Plates, with Lid

NAME

Greiner Bio-One

BRAND

665180

SKU

<https://shop.gbo.com/en/row/products/bioscience/cell-culture-products/cellstar-cell-culture-multiwell-plates/665180.html>

LIN  
K

Polystyrene 12-well cell culture plate with a lid with condensation rings

SPECIFICATIONS

## Equipment

Paintbrush	NAME
Junior Synthetic Bristle Brush Flat (series 329 no. 10)	TYPE
da Vinci	BRAND
DV329/10	SKU
<a href="https://www.gordonharris.co.nz/product/23134-da-vinci-junior-synthetic-bristle-brush-flat-10">https://www.gordonharris.co.nz/product/23134-da-vinci-junior-synthetic-bristle-brush-flat-10</a>	LINK
Flat student grade synthetic bristle paintbrush	SPECIFICATIONS

## Equipment

Paintbrush	NAME
Student Bristle Flat (series 29 no. 12)	TYPE
da Vinci	BRAND
DV29/12	SKU
<a href="https://www.gordonharris.co.nz/product/8453-da-vinci-student-bristle-flat-29-12">https://www.gordonharris.co.nz/product/8453-da-vinci-student-bristle-flat-29-12</a>	LINK
Flat student grade hog bristle paintbrush	SPECIFICATIONS

## Safety warnings

- ! Consult the appropriate safety data sheets and the relevant health and safety procedures before starting.
- Paraformaldehyde (PFA) - carcinogenic, causes serious eye damage, skin sensitisier/irritant, & ecotoxic
  - Ethanol - flammable & an irritant

## Before start

See the guidelines section.

## Dil Preparation

### 1 Make the Dil stock solution (recipe for $\text{40 mL}$ of $[M] 0.15 \text{ mg/mL}$ )



**Figure 1.** Schematic of the Dil stock solution preparation.

#### 1.1 Combine the following in a falcon tube.

- $\text{6 mg}$  of **Dil crystals**
- $\text{40 mL}$  of **100% Ethanol**

#### 1.2 Vortex the solution intermittently for several minutes until no clumps remain.

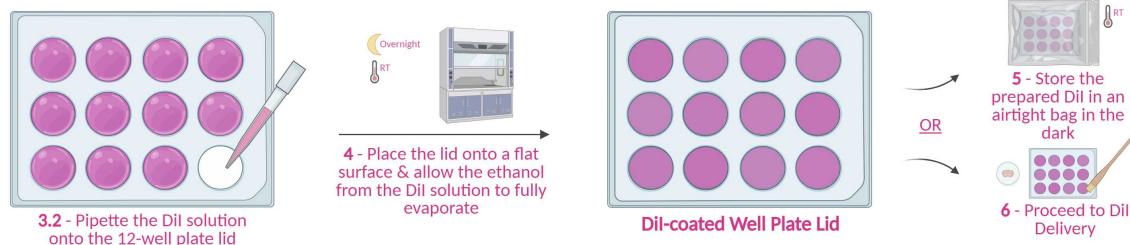
3m

#### 2 **Storage** - Decant the prepared Dil solution into a dark/amber glass bottle, seal it, and wrap it with foil to protect it from light. Then, store the bottle at $4^\circ\text{C}$ until further use.

#### Note

1. If desired, the solution can be split into smaller aliquots and tightly sealed (*e.g. if used infrequently or concerned about evaporation &/or light exposure over time*).
2. The Dil stock solution is relatively stable. We have tested its use for up to 6 months.

#### 3 Prepare the **Dil-coated well plate lids** ( $\text{150 } \mu\text{L}$ of stock solution per well lid $\therefore \sim 0.0225 \text{ mg}$ of Dil per well).



**Figure 2.** Schematic for the preparation of Dil-coated well plate lids.

### Note

1. The **amount of Dil** can be increased by pipetting more of the stock solution or by making a more concentrated stock solution.
2. Also, the Dil can be prepared onto **other well plate sizes**. For example, the lid of a 6-well plate can be used if a larger surface area is desired. The amount of stock solution added will need to be adjusted accordingly.

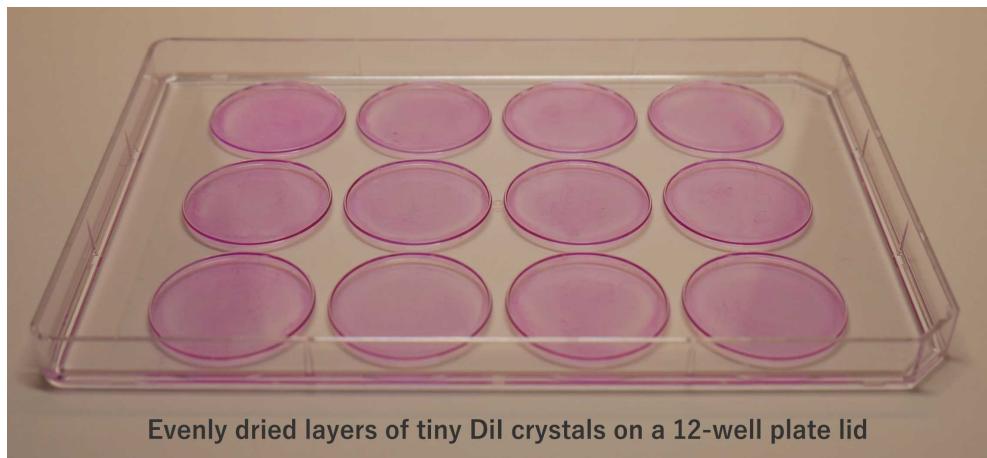
- 3.1 Place a clean, dry lid of a 12-well plate onto a flat surface (interior facing upwards).
- 3.2 Briefly vortex the **Dil solution** & pipette  150  $\mu\text{L}$  onto each well lid. Make sure the Dil solution does not go over the ledge/condensation rings of the well lid. 2m
- 4 Leave to dry overnight on a flat surface in the dark (e.g., in a fumehood or on a bench in a dry room). Avoid disturbing the plate whilst drying; otherwise, it will become uneven. 8h

 Overnight

 Room temperature

## Expected result

Evenly dried Dil results in a uniform layer of tiny crystals!

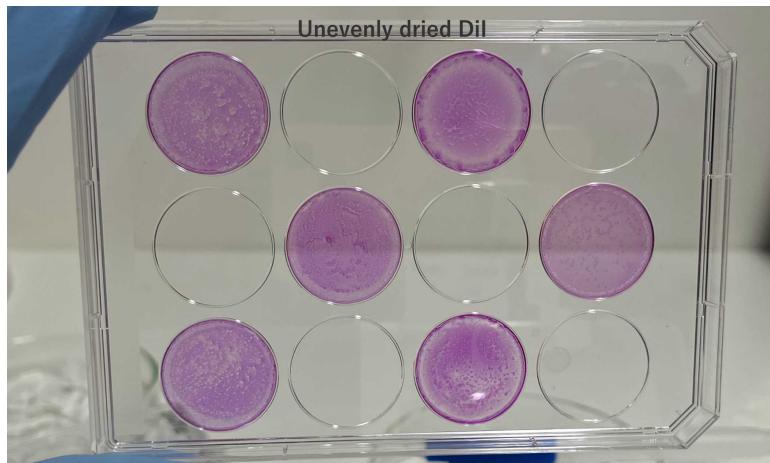


**Figure 3.** Example of evenly dried Dil.

- Proceed to Dil delivery  go to step #6 or storage  go to step #5 if the prepared Dil looks like this.

## Note

Unevenly dried Dil results in clumping!



**Figure 4.** Example of unevenly dried Dil.

- In this case, add 150 µL of 100% ethanol onto each well lid & gently swirl to redissolve the Dil. Then, let it dry as described in  go to step #4

5 **Storage** - Clasp the dried **Dil-coated well plate lid** onto the accompanying well plate, wrap it in foil, and store it in a dry, dark cupboard or drawer at  Room temperature until further use (use within 2 weeks).

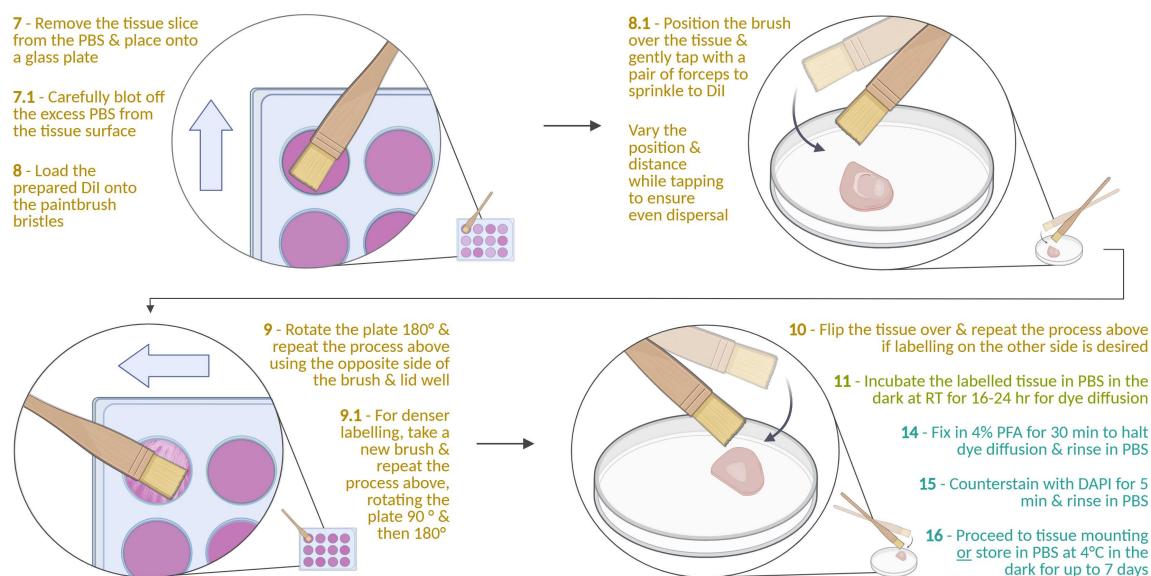
#### Note

1. For **longer-term storage** (*e.g.* > 1 month), place in an airtight ziplock bag or container with a silica desiccant packet.
2. Exposure to moisture prevents Dil transfer & exposure to light causes fading.

## Dil Delivery

5m

6



**Figure 5.** Schematic of the Dil delivery process.

See the video demonstration & refer to the timestamps in the description bar.

<https://www.youtube.com/embed/LtF5g5fkxdM?si=Yvl4wQZtgHyToDbx>

### Note

1. **Tissue dryness** - The tissue shown in the video is a bit overdried due to the extended filming time. Try to work faster, aiming for <3 minutes!
2. **Tapping time** - The video shows the brush being tapped longer than necessary. Most of the Dil is dislodged in the initial taps to the brush - so the time can be reduced as indicated in the steps below.
3. **Labelling pattern** - The video shows broad coverage of the entire slice. Adjust your application pattern to focus on your specific region of interest.

7 Remove the fixed tissue slice from the PBS and place it onto a clean, dry surface (e.g., a glass petri dish or a well plate lid).

7.1 Using a pair of metal forceps, take a small piece of a shredded Kimwipe and carefully blot the excess PBS from the tissue surface via capillary action. Hold the glass dish and tilt it towards yourself to aid in drawing the liquid away. Avoid directly blotting over the region of interest and overdrying it.

### Note

1. **Excess surface liquid** prevents the Dil from penetrating & adhering to the brain tissue.
2. The Kimwipe is shredded into small pieces so no sharp corners can poke the tissue & the jagged edges help draw the liquid away.
3. The tissue slice should still appear a bit shiny and not matte.

8 Take a paintbrush (*see materials section & the note below for more information*) and load the Dil onto one side of the bristle tips by dragging them across half a Dil well lid in a short, smooth stroke. Try to apply even pressure across the bristles and avoid the edges/condensation ring of the well lid.

### Note

1. Moving the bristles back & forth across the well lid will encourage Dil clumping.
2. If the bristles are too flexible, pinch the section close to the ferrule for better maneuverability.
3. Make sure the prepared Dil & the paintbrush are fully dry! Otherwise, the Dil crystals will struggle to latch onto the bristles & also struggle to detach from them (*see figure 7*).

8.1 Position the paintbrush parallel to the tissue slice, about 2 cm above it, with the bristle tips aligned with the slice's top edge. Use a pair of metal forceps to gently tap the brush's ferrule,

10s

dislodging and sprinkling Dil onto the tissue. Between taps, move the brush forward and backward for even dispersion (tap for ~10 s).

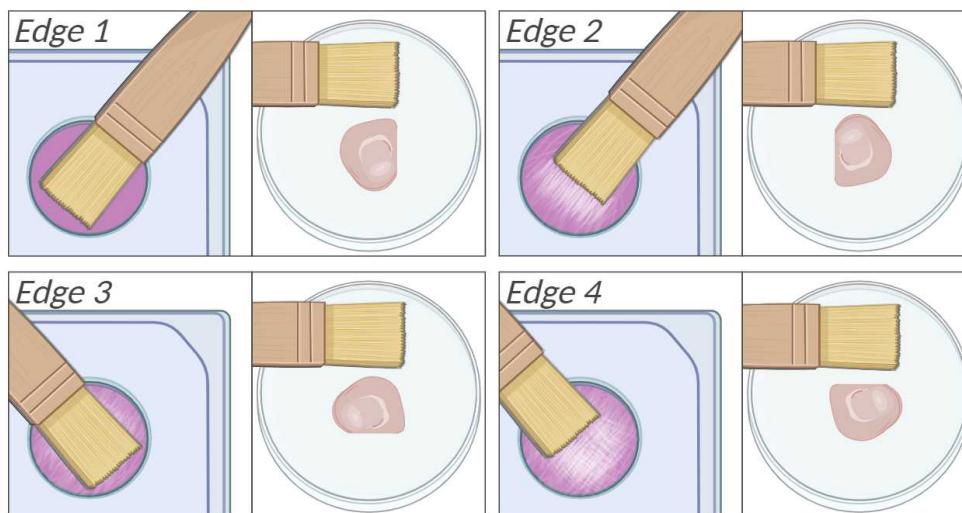
### Note

1. We use a pair of metal forceps to tap the brush because it has a bit of weight behind it. This makes it quite effective at dislodging the Dil from the bristles. However, anything slightly heavy will work, too (e.g. a scalpel handle or metal ruler).
2. Starting off with gentle taps is important; if not, all the Dil will be dislodged at once.

8.2 Next, vary the brush angle and distance (e.g., hold it perpendicular to the slice) and tap more firmly to dislodge the remaining Dil (tap for ~5 s). 5s

9 Rotate the plate 180°. Flip over the paintbrush and drag the unused side of the bristles onto the unused half of the Dil-coated well lid as described in **step 8**  go to step #8 . Then repeat **steps 8.1** and **8.2** (this will be Dil delivery to **edge 2** - see figure 6).

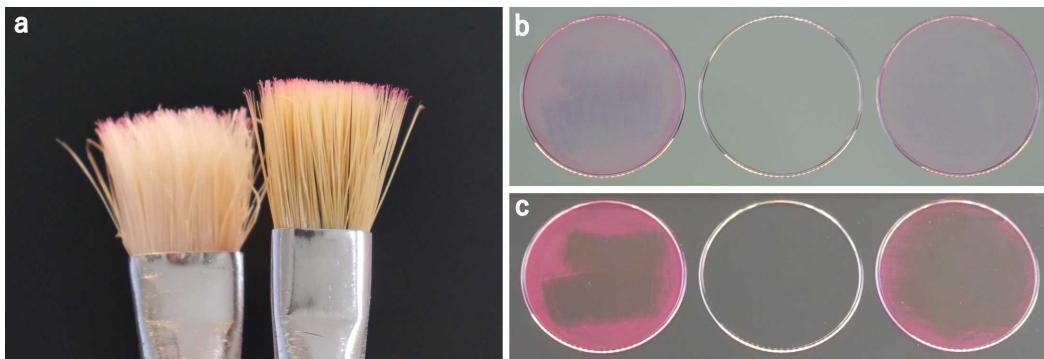
9.1 For more coverage, rotate the slice 90° and then 180°, repeating **steps 8, 8.1**, and **8.2** (this will be Dil delivery to **edges 3** and **4** - see figure 6). Before doing so, either use a new brush or clean the brush by wiping the bristles on a clean Kimwipe and tapping to remove the excess Dil (this is to prevent clumping).



**Figure 6.** Schematic of the Dil delivery sequence (4 edges of the tissue).

## Expected result

The bristles should appear pink & the well lid should appear streaky after Dil delivery!



**Figure 7.** The paintbrush bristles & Dil well lids after delivery. **a)** Pink dye residue on the tips of the bristles. **b)** A used Dil well lid. The unused areas appear as a dusty magenta colour, while the used areas appear as faint purple streaks. **c)** The same well lid under a darker background.

- If not, then it may be that the paintbrush &/or well lids were not dry enough.
- Or, the bristles were too soft &/or sparse to effectively load the Dil crystals.

## 10 OPTIONAL: LABELLING THE OPPOSITE SIDE OF THE TISSUE

Repeat on the other side if desired, but make sure to re-moisten the tissue slice with PBS before picking it up. Also, make sure to flip it over onto a clean section of the glass plate to avoid picking up Dil crumbs dropped from the previous steps.

### Note

1. Most methods do not label both sides because thinner tissue sections tend to be used, e.g. 200-400 µm thick slices.
2. However, we labelled both sides as we used tissue sections that were ~800µm thick & imaged both sides with a non-permanent mounting setup (*see the guidelines for more information*).

## Dil Incubation

20h

- 11 Return the tissue slice to the PBS in the 12-well plate. Cover and incubate for 16-24 h at RT in the dark to allow the dye to diffuse along the neuronal arbours.  20:00:00 16-24 h

 Room temperature

## Note

1. The **dye's diffusion speed** depends on various parameters, but mainly on tissue fixation. Our incubation duration is based on brain tissue fixed in 1.5% PFA.
2. Dye diffusion is much faster in unfixed samples, and shorter incubation times are used (*see the guidelines section for more information*).

## 12 **OPTIONAL: CHECKING THE DENSITY OF LABELLING** (1-2 h after Dil delivery)

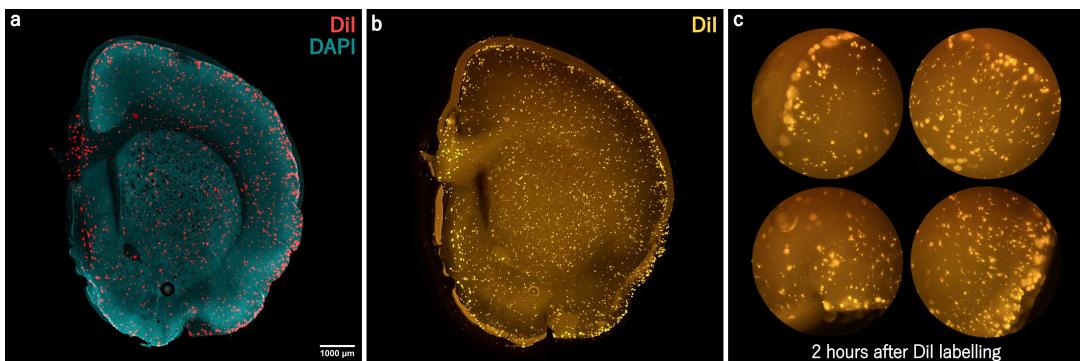
Carefully remove the labelled slice and mount it onto a glass-bottom imaging dish with plenty of PBS to avoid drying whilst viewing. Afterwards, return to the well and resume the incubation process.

30m

- If the **labelling is too sparse**, the slice can be relabelled by repeating the processes above. However, the additional handling may damage the tissue, so we typically do not relabel.
- Instead, we recommend first becoming proficient with the delivery technique using test samples. Do this by carefully documenting the Dil delivery process and the resulting labelling pattern, then repeating until the desired pattern is consistently obtained. Each time, make small adjustments to the technique and then see how it affects the pattern of neuronal labelling.
- Once mastered, you will be able to perform the Dil delivery with the unaided eye and achieve consistently high-quality labelling. You will not need to perform labelling checks or relabel the tissue.

## Expected result

Ideally, you should see a dense pattern of tiny Dil spots distributed across the slice!



**Figure 8.** Coronal brain slice 2 hours after Dil labelling. The labelled slice was imaged with an inverted Olympus FV3000 laser scanning confocal microscope (CLSM). **a)** 1.25× objective lens in laser scanning mode (LSM). **b)** 1.25× objective lens in ocular mode. **c)** 10× objective lens in ocular mode. The ocular mode images were acquired using a cellphone camera attached to the eyepiece via a 3-D printed **adaptor**. Note that some spots appear diffuse because they are out of focus rather than damaged.

1. The pattern of Dil dispersion is comparable to that obtained with DiOlistic labelling. For a comparison, see Bevan et al. (2024), Wu et al. (2004), and Seabold et al. (2010).
2. At **2 hours**, neuronal labelling is incomplete, but **signs of successful uptake** should be clear. For example, neuronal projections should appear as **small directed streaks radiating from the dye spot**. However, not every spot will result in successful neuronal labelling. For example, some will land in the neuropil and result in a more diffuse spread. Or some will be too clumpy.
3. If the majority of the dye spots appear as **small, intensely saturated spots**, then the Dil crystals may not have been successfully incorporated into the tissue because there was too much liquid on the surface during Dil delivery.
4. If most dye spots appear as **diffuse blobs**, then the tissue was likely damaged at some point.

## Dil Post-processing

1h

- 13 Bring the following solutions to Room temperature

15m

- 4% PFA
- PBS
- DAPI

## Note

1. Drastic temperature changes can disrupt dendritic morphology (*e.g. solutions that are too cold*).
2. Preparing the solutions as small aliquots speeds up this process.

14 Under the fumehood, remove the excess PBS from the well containing the labelled tissue and add  3.5 mL of 4% PFA. Incubate for 30 min.

30m



## Note

The post-fixation step impedes further dye diffusion. This does not completely stop it but drastically slows it down, which is important for maintaining crisp & clear neuronal labelling & prolonging the visualisation period.

## Safety information

*Use the appropriate PPE & follow the proper handling/disposal procedures when using PFA!*

14.1 Remove the PFA and rinse twice with PBS (1 min per wash).

2m

15 Add  400 µL DAPI solution to the well. Tilt the plate while incubating to ensure the solution fully covers the tissue (otherwise, use more DAPI solution or use smaller volume well plates, e.g. a 24-well plate).

5m

## Note

1. DAPI nuclei stain is used to visualise the neuroanatomical boundaries & assess the quality of the tissue slice. Other nuclei stains like Hoescht can be used if desired.

15.1 Remove the DAPI and rinse twice with PBS (5 min per wash).

10m

16 Either proceed to **storage** or **tissue mounting**.

- For **storage**, place into a well filled with PBS, cover and wrap in foil, and store at 4°C.
- For **tissue mounting**, refer to the guidelines section for further information.

#### Note

1. Be mindful of the **refrigerator** used for **storage**. Refrigerators with variable temperatures, such as freeze-thaw cycling, compromise the membrane integrity & the quality of the Dil labelling.
2. If available, store the tissue in a **lab-grade refrigerator** with consistent, well-regulated temperatures. If not, it may be best to proceed immediately to tissue mounting & imaging.
3. For dendritic spine imaging, we find the **optimal time frame** for image acquisition to be between 24 hours to 4 days, while the **visualisation limit** is around 7 days after Dil labelling. This may be longer for larger features of interest, such as dendritic branching patterns.

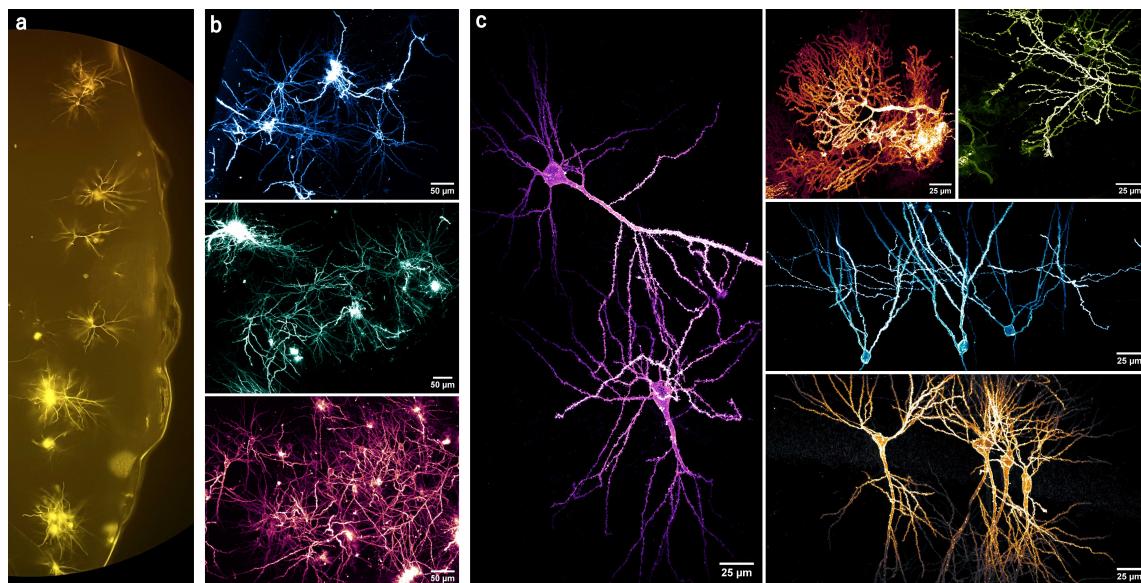
## Cleaning & Maintenance

5m

- 17 To **clean the paintbrushes**, fill a small container with ethanol and dip the Dil-coated bristles into it. Brush against the walls of the container to break up any remaining Dil and drag across a Kimwipe. Repeat this process once, then allow the brush to air-dry for 24 hours before reuse.
- 18 To **clean a used Dil-coated well plate lid**, pour ethanol over it and allow the Dil to dissolve. Wipe the lid clean with a Kimwipe, and repeat if necessary. Then rinse with ddH<sub>2</sub>O and air-dry before reuse.

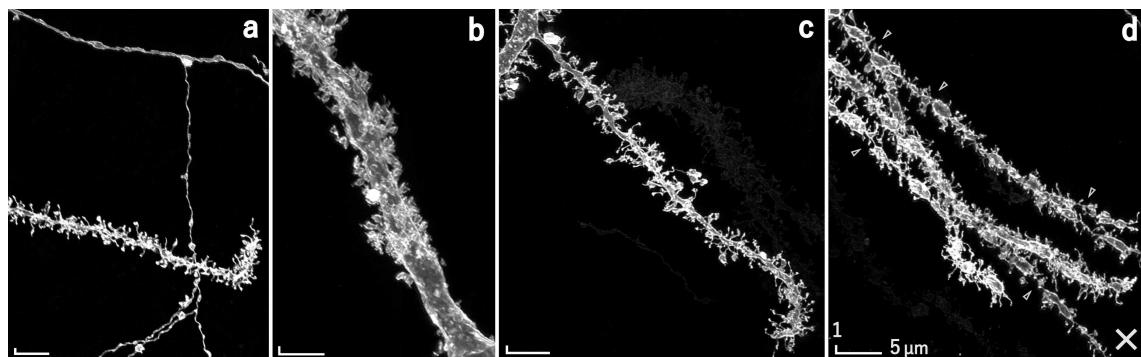
## Examples

- 19 **Examples of whole neurons**



**Figure 9.** Examples of neurons labelled with the Dil paintbrush method. Images were acquired using an Olympus FV3000 CLSM. **a)** A patch of cortical pyramidal neurons labelled with Dil taken with a 10x objective lens in ocular mode. The image was acquired by mounting a cellphone camera to the eyepiece with an [adaptor](#). **b)** Lower-quality image stacks of neuron patches acquired with a 20x objective lens in LSM mode. The intensely saturated, irregularly shaped white spots are where the Dil crystals have landed. **c)** Higher-quality image stacks of whole neurons acquired with a 60x silicone oil immersion objective lens. The image stacks in **b)** & **c)** are maximum intensity projections (MIP) displayed with look-up tables (LUTs) from the [KTZ set colorful black to white](#) via [NeuroCyto LUTs](#) in [ImageJ](#).

#### Examples of dendritic segments



**Figure 10.** Examples of dendritic segments. Image stacks were acquired using an Olympus FV3000 CLSM. **a)** A terminal basal dendritic segment covered in spines & an axon with boutons from a cortical pyramidal neuron. **b)** Thorny excrescences from a dendritic segment of a hippocampal CA3 pyramidal neuron. **c)** A terminal apical tuft from a cortical pyramidal neuron densely covered in dendritic spines with complex morphologies. **d)** Dendritic segments from extremely truncated cortical pyramidal neurons. The arrows indicate the notching. Truncated dendritic segments at the tissue surface often show aberrant notching (& possibly swelling), similar to dendritic varicosities seen after neuronal injury. These truncated surface segments appear to provide "high-quality" image stacks due to intense labelling & less axial blurring compared to the deeper segments from intact neurons. Although tempting to sample, these truncated segments should be avoided as they introduce confounds. All images were acquired with a sampling pixel of 50 nm & z-step of 150 nm using a 60x silicone oil immersion objective lens & then deconvolved in CellSens using either 25 iterations of the fast maximum likelihood algorithm (a) or 20 iterations of the advanced maximum likelihood algorithm with noise reduction (b,c,d). Following deconvolution, the z-series were stacked in ImageJ & presented as greyscale MIP with a 1 & 5 μm scale bar.

1. Figures **9a** & **9b** illustrate the variation in the neuronal labelling patterns and sizes of the Dil crystals/spots produced by this method. Overall, a large population of neurons are randomly labelled.
2. This method's labelling pattern allows the neuronal morphology to be clearly observed, as depicted by the diverse types in figure **9c**. Moreover, the order and origin of the dendritic branches can be easily traced back to the soma, making it suitable for dendritic analyses (see figure **10**).
3. Of course, not every dye spot on the tissue results in neuronal labelling, perhaps due to Dil clumping, failure to incorporate into the tissue, tissue damage, or landing in the neuropil, etc. And not every labelled neuron will be suitable for sampling due to truncation (see figure **10d**), insufficient labelling, perpendicular projections or projections beyond the working distance, being obscured by other features &/or cells, etc.
4. The sampling criteria will differ depending on the features and analyses of interest. For example, dendritic spine imaging is rather complex and warrants further considerations that are beyond the scope of this protocol (see the literature listed in the guidelines for more information).
5. Nevertheless, this method labels a large population of neurons, with plenty suitable for sampling. The following literature provides a good starting point for guidelines on sampling

- Dickstein et al. (2016), Dumitriu et al. (2011), and Wu et al. (2004).

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