**TITLE:**

Labeling Neuronal Morphology Using Custom Diolistic Techniques

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**SHORT ABSTRACT:**

Here, we present a protocol for custom diolistic labeling. The customization of this fluorescent neuronal labeling method provides a modifiable technique that can be adapted to a wide variety of research goals and applications in the analysis of neuronal morphology.

**LONG ABSTRACT:**

Diolistic labeling is increasingly utilized in neuroscience as a highly efficient and reproducible method for the visualization and analysis of neuronal morphology. The use of lipophilic carbocyanine dyes, combined with particle-mediated biolistic delivery, allows for the non-toxic fluorescent labeling of multiple neurons, including their dendritic arbors and spines, in both living and fixed tissue. Since first described, this novel labeling method has been modified and adapted to fit a variety of research goals and laboratory settings. Diolistic labeling has traditionally relied on the use of a commercially available, hand-held gene gun for the propulsion of coated micro-particles into tissue sections. Recently, laboratory-built biolistic devices have been developed and allow for the increased availability and customization of this method. Here, we discuss one such custom biolistic device and provide a detailed protocol for its use in diolistic labeling. In addition to decreasing the associated costs, the laboratory-built device also overcomes many of the obstacles normally experienced with traditional diolistics, allowing for reliable and reproducible neuronal labeling. The versatility of this method allows for its adaptation to a variety of laboratory settings and neuroscience-related research goals.

**INTRODUCTION:**

Three-dimensional morphological reconstructions of individual neurons and their dendritic arbors have served as the bases for analyzing the structure-function relationships within the nervous system1-3. For over a century, the main method for these studies consisted of various modifications of the famed Golgi staining procedure4, which has proved invaluable in developing our modern understanding of the nervous system1,2. However, this method and the various modifications of the silver impregnation of a percentage of the neuronal population are not without drawbacks. In particular, the small population of stained cells, while an advantage in morphological studies, elicits the lingering concern of selection bias2,5. The Golgi method also suffers from a limited compatibility with modern immunolabeling techniques and confocal microscopy. Additionally, data analysis following the Golgi method of staining may be negatively influenced by inconsistent neuronal impregnation, sectioning artifacts, and the overlapping of fine dendritic processes that leads to an indistinguishable morphology.

More recently, the use of neuronal transfection and electroporation methods have allowed for neuronal morphological labeling that circumvent the limitations experienced with Golgi staining6,7. While the administration of dye into individual cells through microinjection using intracellular or patch pipettes generates excellent single-cell labeling, the technique is technically demanding and may be vulnerable to sampling bias6,8-10. Neuronal transfection relies on the introduction of DNA constructs into target cells and tissues through a variety of methods. One such method of DNA transfer is known as “Biolistic” delivery, in which a gene gun utilizes a pressurized release of gas to propel DNA-coated micro-particles into tissue, crossing the plasma membrane to target the cells3. This method does not require the same level of technical expertise needed for conventional intracellular injections11. Furthermore, the random sampling used in biolistic delivery is ideal for full-scale quantitative analysis. However, the reliance on DNA transfection for fluorescence expression limits the method to use on living tissues in prepared cultures.

A more recent advancement from the biolistic approach to the morphological labeling of live or fixed neurons was first reported by Gan *et al.* in 200012 and is known as diolistic labeling. Diolistic labeling utilizes the lipophilic fluorescent dye dialkylcarbocyanine (DiI). While DiI has traditionally been used in anterograde and retrograde neuronal tracing, it has also proven to be effective in the fluorescent labeling of the neuronal cell membrane. Utilizing a diolistic approach13, the ballistic delivery of DiI-coated micro-carriers to fixed or cultured tissue slices allows the DiI to be incorporated into the cellular membrane. This occurs through lateral diffusion in living or fixed tissue, illuminating the cellular morphology. When ballistically delivered to tissue sections through a single pulse of high-purity gas (*i.e.,* helium or nitrogen), individual DiI-coated tungsten particles are embedded in various neurons of the tissue. The micro-carriers pass into the soma of the neuron, while the DiI is captured in the neuronal membrane. Thus, the DiI is allowed to diffuse along the cellular membrane of a single neuron, fluorescently labeling the fine neuronal architecture of dendritic branches and spines13. DiI-labeled neurons can be observed through high-resolution imaging, such as confocal or two-photon microscopy, and can be digitally reconstructed in precise detail. The quantification and classification of dendritic branching and dendritic spines can be accomplished with appropriate software packages14,15.

Since its development, diolistic labeling has relied on the use of a commercially available gene gun, designed for biolistic transfection, for the propulsion of dye-coated micro-particles into tissue. However, these handheld devices have several drawbacks in the proposed staining method. First, the handheld nature of the device can alter the exact angle of delivery of the particles, causing inconsistent dye patterning in the tissue. Next, the standard tubular barrel of the gun causes increased particle density in the middle of the field and produces a burst of gas strong enough to damage the superficial layer of fixed tissue. Finally, the cost of commercially available devices and the associated materials may preclude some laboratories from using this type of methodology. In an effort to circumvent the aforementioned obstacles, Bridgman *et al.*11 designed and constructed a custom-built device for use in biolistic applications (Figure 1). With protocol modifications, the device has been optimized for the diolistic labeling of fixed tissues. The device consists of a solenoid valve triggered by a relay switch to fire for 50 ms and is a modification of an original model constructed by Dr. David Kirk at Washington University in Saint Louis. The main features of the device that contributes to its reliability and reproducibility include a precisely timed trigger for the solenoid valve, a precise height adjustment system, a narrow baffled barrel, and a small pore size filter11. Since the gun is mounted on a fixed base, the angle of delivery remains the same throughout all procedures, while the baffled barrel (constructed based on the design described by O’Brien)14 limits the amount of pressurized gas that contacts the tissue. Particle carriers (cartridges) are supported by a plastic ring that fits inside a modified filter holder (cartridge holder)11. The particle carriers are cut-off yellow (200 µL) pipette tips. The device components listed here allow for a similar device to be constructed and operated with commercially available materials for a lower cost than traditional gene gun systems.

**PROTOCOL:**

All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals. This protocol has been approved by the IACUC at Missouri State University.

Note: The following protocol will detail the methods used in the diolistic labeling of fixed brain tissue obtained from rats prepared using transcardial perfusion. Previous studies16 have utilized similar methods of labeling but without the transcardial perfusion and from various animal species.

1. **DiI/Tungsten-Coated Bead Preparation**

Note: The DiI (1-1'-Dioctadecyl- 3,3,3',3'-tetra methylindo carbocyanine perchlorate)-coated beads should be prepared at least 72 h in advance of tissue preparation to allow for adequate drying and optimal labeling. Prior reports1 have shown increased success using CM-DiI when performing additional immunolabeling.

* 1. Place a clean glass slide on waxed weigh paper under the fume hood.
  2. Add 90 mg of 1.3-µm tungsten powder to a glass slide and dice thoroughly using a clean razor blade. Spread the fine powder evenly over the slide in a thin layer free of clumps.
  3. Dissolve 2 mg of DiI with 75 µL of methylene chloride in a 200-µL microfuge tube under the fume hood. Seal the tube quickly.

CAUTION: Methylene chloride is caustic and evaporates extremely quickly; care should be taken to work under the fume hood and to minimize the time uncovered.

* 1. Vortex the microfuge tube to ensure that the DiI dissolves completely.
  2. Slowly release the DiI/methylene chloride mixture over the surface of the 90 mg of evenly-spread, 1.3-μm tungsten particles on the glass slide, ensuring that the DiI is applied evenly across the tungsten particles. The pipette tip can be used to ensure the even mixing of the tungsten particles and the DiI solution.
  3. Allow the methylene chloride to dry under the hood until the bead mixture turns a light gray. Using a single-edged razor blade, scrape the DiI-coated tungsten particles loose from the slide. Continue to chop the DiI/tungsten particles evenly across the glass slide until the particles appear as a fine powder.
  4. Scrape the coated tungsten beads carefully onto weigh paper and transfer them to a 15-mL conical tube containing 10 mg/mL PVP in distilled water. Vortex vigorously for 30 s.
  5. Sonicate the DiI/PVP solution for 10 min in a water bath at room temperature.

1. **Cartridge Preparation**

Note: Cartridges are made using standard 200-µL yellow pipette tips. Low-retention pipette tips should not be used in this protocol, as they may interfere with particle adherence to the inner wall of the tip. The protocol outlined here utilizes standard polypropylene research-grade pipette tips throughout the testing.

* 1. Immediately following sonication, vortex the DiI/PVP solution vigorously for 10-20 s.
  2. Pull 100 µL of the DiI/PVP solution from the middle of the conical tube into a mounted pipette tip cartridge.
  3. Hold the pipette in a horizontal position, parallel to the ground, and rotate it slowly over 360°.

Note: Some particles suspended in the solution should attach to the pipette tip inner wall in an even fashion. Rotating too quickly or too slowly will affect the adherence of particles to the wall.

* 1. Slowly release the PVP solution and unbound particles back into the conical vial and gently disconnect the pipette tip from the pipette while continuing a slight axial rotation. Lay the pipette tip horizontally on the benchtop and avoid any unnecessary disturbance.
  2. Re-vortex the original DiI/PVP solution for 10-20 s and repeat the procedure (steps 2.1-2.4) with the next cartridge. Repeat the procedure until the DiI/PVP solution appears relatively clear (typically ~25 cartridges are prepared).
  3. Gently remove the cartridge/pipette loading end and 10 mm of the tip using a single-edge razor blade, revealing ~20 mm of the middle of the cartridge/pipette. Determine the successful loading of the cartridge by confirming the presence of a light haze of tungsten beads adhering to the inside wall of the cartridge, without the presence of large aggregations or clumps of beads. Discard any unsuccessful cartridges.
  4. Protect the cartridges from light and store them with a desiccant for a minimum of 72 h and a maximum of 1 month if stored at 4 °C.

1. **Tissue Preparation**

Note: Through repeated trials, it has been found that tissue fixation by the transcardial perfusion of a low-concentration (1.5%) aldehyde prepared with 25 mM PBS greatly enhances the results of neuronal labeling. Higher concentrations (4%) were determined to over-fixate the tissues and result in incomplete labeling. Lower fixative levels resulted in excessive dye leakage from cellular membranes.

* 1. Anesthetize the animal and confirm deep anesthesia through an IACUC-approved method. Open the abdominal and thoracic walls, exposing the peritoneal and thoracic cavities.
  2. Transcardially perfuse through the left ventricle with ice-cold 25 mM PBS (pH 7.2) until a clear perfusate consistently returns through the opened right atrium; the clearing of the liver (*i.e.,* a color change from dark red to pale brown) also indicates a successful exsanguination.
  3. Transcardially perfuse with ice-cold 1.5% paraformaldehyde until the neck and hindquarters are rigid.

Note: The times and amounts of fixative for the perfusion may vary between labs and between the species, age, and size of the animals.

* 1. Carefully remove the brain by exposing the skull and removing the calvarium with rongeurs. Take care to avoid any unnecessary or direct handling of the brain tissue.
  2. Postfix the intact brain for 2 h in 1.5% paraformaldehyde.
  3. Prepare regions of interest or whole-brain specimens under a dissecting scope, taking care to prevent tissue drying.
  4. Prepare a 4% solution of low-melting point agarose in PBS. Heat the agarose until molten with constant stirring to avoid excessive bubble formation.
  5. Pour the molten agarose into an appropriately sized histology embedding mold for the brain tissue and keep it on a 40 °C warming plate. Orient the brain tissue in the desired plane for sectioning and place it in the molten agarose. Remove the agarose from the heat and allow it to cool to room temperature before handling.
     1. Avoid placing it in a refrigerated location to speed cooling, as this will negatively affect the embedding and may cause the agarose to break away from the tissue during sectioning.
  6. Remove the cooled block from the mold and trim the excess agarose from the tissue, making sure that all areas of the brain tissue are surrounded by at least 5 mm of agarose.
  7. Secure the tissue block to a mounting block using a cyanoacrylate glue and allow it to dry for a few minutes.
  8. While drying, prepare culture plates by filling each well with 2 mL of PBS.
  9. Place the mounting block onto the vibratome stage and secure it. Lower the stage completely. Attach a clean blade, free of any preserving oils.
  10. Fill the vibratome chamber with cold 25 mM PBS to the specified level so that the blade is covered.

Note: Only PBS should be use to fill the vibratome well. Lower concentrations or pure water will cause the tissue to swell.

* 1. Trim the block in 300-µm increments until the desired section depth is reached.
  2. Vibratome-section the brain into 150- to 300-μm serial sections throughout the region of interest. A high amplitude (8.5 Hz) and low speeds (0.5) are recommended for sectioning brain tissue but should be tested on the vibratome in each laboratory.
  3. Using a paintbrush, remove the sliced tissues from the PBS vibratome bath and transfer them into the tissue culture dishes containing PBS. Store them until the DiI labeling is to be performed.

Note: Prior experiments have found that tissues may be stored at 4 °C for up to one week prior to labeling without significant impact on tissue labeling1,17.

1. **Diolistic Cellular Labeling**
   1. Carefully remove previously prepared DiI/tungsten-coated cartridges from the storage container and load them into individual cartridge holders.
   2. Load each individual cartridge holder into the device and set the gas pressure to 120 psi using the regulator.

Note: The device presented here uses high-purity compressed nitrogen as a propellant, but helium may be used instead.

* 1. Place the culture plate under the device barrel and carefully pull off the PBS using a pipette. Take care to avoid damage to tissue, and ensure that the tissue lays flat on the bottom of the culture plate. Adjust the position using a paint brush, if needed.

Note: Fluid left covering the tissue can cause beads to ricochet from the surface, negatively impacting tissue labeling.

* 1. Place a 3.0-µm microplate filter insert over the tissue and lower the barrel to a set point 15 mm above the tissue surface. Avoid handling the filter inserts with ungloved hands, as dirt and oils may clog the filter.
  2. Activate the device using a relay switch that allows a 50-ms burst of gas to pass through the device and cartridge.
  3. After the shot, immediately raise the barrel and remove the filter insert. Return the PBS to the well, being careful not to damage the tissue. Take care to ensure that the tissue surface, vertical at the time of labeling, remains vertical for the remainder of the procedure; this is especially important for thicker tissue sections.
  4. Load a new cartridge into the device and repeat the process (steps 4.1-4.6) for each tissue section in the culture plate.
  5. Once completed, protect the entire culture plate from light and allow it to incubate for 24 h at room temperature.

Note: Through testing, it was determined that room temperature (20-23.5 °C) was ideal to allow the diffusion of the dye throughout all lipid neuronal membranes of all tested neuronal populations.

* 1. Post-fix the sections for 1 h in 2 mL of 4% paraformaldehyde; this step has proven to be useful in minimizing “overstaining,” or the leaching of dye out of neuronal membranes. Wash briefly with PBS and store in fresh PBS at 4 °C after post-fixation.

Note: Tissues can remain stored at 4 °C for up to a week before mounting but should be mounted and imaged as soon as possible for best results.

* 1. Mount tissue slices on coated or charged slides and coverslip with an appropriate antifade agent (5% n-propyl-gallate).

Note: If time allows for imaging in the 24-72 h post-labeling, wet-mounting the slide in PBS and sealing the edges with nail polish or another sealant provides a cost-effect and time-sensitive mounting method. Wet-mounted tissues should be stored in humid chamber at 4 °C when not undergoing microscopy.

1. **Confocal Microscopy and Analysis** 
   1. Image the mounted slides with any confocal microscope equipped with a motorized z-stage and lasers between 543 and 561 nm.

Note: The use of epifluorescence microscopy equipped with TRITC filters will permit the visualization of the DiI-impregnated neurons and can be an important tool in locating areas of interest. However, the use of epifluorescence microscopy in the quantitative three-dimensional morphological analysis of neurons is discouraged. Confocal microscopy allows for increased resolution in images of DiI-impregnated tissues and for optical sectioning through the cells of interest. Digital reconstructions of the optical sections can be assembled into a three-dimensional model for the analysis of morphology1. The representative results presented in this report were obtained using sampling parameters detailed by Staffened and Meisel, 20111. It is recommended that sampling parameters and experimentally/statistically appropriate cell numbers and dendritic segments be established before imaging. There are a variety of commercially available software extensions that can be used for image analysis and morphological reconstructions. These product choices should be based on individual laboratory needs.

**REPRESENTATIVE RESULTS:**

Using the procedure presented here, custom diolistic labeling has been used to characterize alterations in neuronal morphology of the lateral/dentate nucleus of the cerebellum. Here, we show representative labeling results from developing rat pups exposed to abnormally high levels of the serotonergic agonist 5-methyloxytryptamine (5-MT), both pre-and postnatally18. With the aid of quantitative software, dendritic branching morphology (Figure 2), architecture (Figure 3), and synaptic connections (Figure 4) were analyzed.

Successful diolistic labeling can be rapidly confirmed through cursory visualization with bright-field and epifluorescent microscopes equipped with red (TRITC) filter (Figure 5). Areas of interest (Figure 5A and B) and potential cells for imaging can be identified (Figures 5C, D, and E), though morphological details are often obscured prior to confocal imaging. Unsuccessful labeling (Figure 6) can be caused by improper bead and cartridge preparation or by storage, and care should be taken during steps 1 and 2 of this protocol. Over-labeling (Figure 6) is commonly caused by the aggregations of coated beads in one area of the cartridge (step 2.6) and can be minimized by using the proper technique or by decreasing the bead concentration in the slurry solution (step 1.7). Following confocal image acquisition, max projection z-stacks can be quickly obtained through the use of the open-source image processing resource, ImageJ (NIH), and used to evaluate the criteria for acceptable labeling (Figure 7).

**Figure 1: Custom biolistic device.**

Key features include a baffled barrel to improve particle delivery, a knob for height adjustment, and a fixed base. The firing mechanism consists of a solenoid valve activated by a relay switch that allows a burst of nitrogen gas to pass through the cartridge and filter assembly and into the tissue specimens.

**Figure 2: DiI-labeled neuron of the lateral/dentate nucleus of the rat cerebellum.**

A Z-projection of DiI-labeled neurons are used to illustrated the dendritic morphological analysis. The inset image illustrates the three-dimensional computer tracing models used for the quantification of the morphology. A morphometric software package was used to contour the dendritic processes of the digitized 2-D images and to develop three-dimensional models.

**Figure 3: Sholl analysis and a comparison of dendritic branching patterns.**

Significant differences in branching morphology were observed in experimental (5-MT) groups compared to control (saline) populations. (\*\*) denotes a significance value of p ≤ 0.001, (\*) denotes a significance value of p ≤0.05. This figure has been modified from Hough and Segal 201618.

**Figure 4: Analysis of the total dendritic spine numbers and dendritic spine density.**

The mean total number of spines per neuron per group (A)18 and the mean dendritic spine density per group (B)18. Representative DiI-labeled dendritic segments used for analysis of control (C) and experimental (D) groups. (\*\*) denotes a significance value of p ≤ 0.001, (\*) denotes a significance value of p ≤ 0.05. This figure has been modified from Hough and Segal 201618.

**Figure 5: Bright-field and epiflorescence overview of tissue sections and an assessment of cytoarchitectural boundaries18.**

Composite image (A) of low-magnification bright-field photomicrographs. The lateral/dentate nucleus of the cerebellum (LN) (B) (solid arrow) can be viewed at the lateral aspect of the deep cerebellar nuclei, surrounded laterally by white matter. The medial aspect of the LN abuts the dorsolateral hump (DL) (dashed arrow) of the anterior interposed (AI) nuclei (dotted arrow), with the superior cerebellar peduncle (arrowhead) extending from the hilus of the LN. Epifluorescence photomicrographs at low (10x) (C), medium (20x) (D), and high (60x) (E) magnifications. A single LN neuron (arrow) (C and D) is illuminated with the TRITC filter and can be further imaged using the confocal microscope. High magnification (E) resolves some dendritic segments that extend beyond the focal plane (arrowhead), illustrating the need for confocal imaging at a specific z-depth. The LN is outlined in white (C). This figure has been modified from Hough and Segal 201618.

**Figure 6: Representative over-labeling.**

Composite image (A) of low-magnification bright-field photomicrographs representative of failed (over-)labeling with tungsten microparticles (arrow). DiI staining and over-labeling can be visually appreciated by the presence of the pink (arrowhead) coloration outside of a single cell. The epifluorescence photomicrograph (B) shows the over-labeled areas (arrows), with indistinguishable cellular structure.

**Figure 7: Retrograde versus anterograde labeling.**

A laser scanning confocal photomicrograph z-project of LN neurons, representative of retrograde (A) and anterograde (B) DiI labeling. Note the differential appearance of the cell soma (white arrow) with (A) appearing shadowed and incomplete compared to the soma pictured in (B). Care should be taken to avoid including retrograde-labeled neurons in the quantitative analysis if possible.

**DISCUSSION:**

Here, we demonstrate a method of custom diolistic labeling to quantitatively analyze neuronal morphology and synaptic connectivity. The versatility of this method allows for its adaptation to a variety of laboratory settings and research goals. While the results presented here exhibit its use in rat neural tissue, other studies have used diolistic labeling to investigate diverse species through various neuroscience-related applications16. The method is relatively fast, as it takes 1-2 days from tissue fixation to image acquisition and has shown promise in its ability to be combined with other, more classical labeling approaches, such as immunostaining16,19. With a laboratory-designed and -built device, researchers can take advantage of low-cost materials, which may allow for the method to be incorporated into budget-conscious investigations. Material availability may also assist with increasing the number of tissue sections labeled, in turn increasing the cell numbers used in the analysis while decreasing the effects of failed labeling on a particular study.

While diolistic labeling has been shown to be a reliable and reproducible method of dendritic labeling, failures in labeling have been reported. To mitigate these failures, it is important to limit variables in cartridge preparation and storage while outlining clear selection criteria for the inclusion of stained neurons for analysis. Neurons suitable for morphological analysis should: (1) be contained within a region of interest, as defined by cytoarchitectural boundary criteria; (2) demonstrate complete fluorescent labeling of well-defined, tapered dendritic endings; and (3) show primary and secondary dendritic trees with clear branching points. To select individual dendritic segments for imaging and spine analysis, the segment should satisfy the following criteria: (1) the entire dendritic segment, as assessed by tapered, well-defined endings, should fall within the optical depth of the tissue section; (2) dendritic segments should be either parallel or at acute angles to the plane of the section to allow for visualization; and (3) segments should not be obscured by the overlap of other dendritic segments at the same z-depth, which may interfere with the quantification of dendritic spines18,20. By setting criteria such as these, the impact of sampling bias and variations in DiI labeling can be minimized.

The most critical step in this protocol is the preparation of the cartridges. Properly prepared cartridges should only exhibit a light haze of beads adhering to the inner wall of the cartridge. Large aggregations of beads lead to the overstaining of the tissue. Additionally, the cartridges should be adequately stored: protected from light, under desiccation, and at a cool temperature. High temperatures can cause the dye to separate from the tungsten bead, while light will decrease the fluorescence of the dye. Harsh handling of the dried cartridges can cause the “dust” of coated beads to fall from the cartridge prior to shooting, resulting in sparse labeling. Current limitations of this method exist in the truncated timetable from labeling to imaging. While an antifade mounting medium can preserve the labeling for extended times, these times pale in comparison to those of other neuronal labeling methods, such as Golgi staining. Experimentation on prolonging tissue labeling by using submersion in antifreeze solutions and storage in sub-zero temperatures is currently underway, with positive preliminary results. Secondary limitations in the method concern the ability to precisely target neuronal populations in a large tissue sample. The device is currently designed to provide a sparse labeling pattern, uniformly spread over a tissue. This approach limits the ability of an investigator to selectively target a population of cells with any accuracy. Future advancements in the firing mechanism and micro-barrel design may facilitate adjustments and refinements in aiming and selection in the future.

The diolistic approach to fluorescently label neurons in their entirety has many advantages when compared to more traditional techniques. The rapid nature of the method, the passive diffusion of the dyes, and the compatibility with confocal microscopy provide an unparalleled opportunity to examine features of neuronal cells at a high spatial resolution in a complex, three-dimensional tissue environment. Diolistic labeling should be viewed as a modifiable technique that can be customized to individual research goals and applications.

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**DISCLOSURES:**

The authors have nothing to disclose.

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