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Labeling of Neuronal Morphology using Custom Diolistic Techniques
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Abstract:	Diolistic labeling is increasingly utilized in neuroscience as a highly efficient, reproducible method for visualization and analysis of neuronal morphology. The use of lipophilic carbocyanine dyes, combined with particle-mediated biolistic delivery allows for non-toxic fluorescent labeling of multiple neurons with 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 which allow for increased availability and customization for this method. Here, we discuss one such custom biolistic device and provide a detailed protocol for its use in Diolistic labeling. While decreasing the associated costs, the laboratory built device also overcomes many of the obstacles normally experienced with traditional diolistics to allow 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.
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1 **TITLE:**
2 Labeling Neuronal Morphology Using Custom Diolistic Techniques
3
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22

23 **SHORT ABSTRACT:**
24 Here, we present a protocol for custom diolistic labeling. The customization of this fluorescent
25 neuronal labeling method provides a modifiable technique that can be adapted to a wide
26 variety of research goals and applications in the analysis of neuronal morphology.
27
28 **LONG ABSTRACT:**
29 Diolistic labeling is increasingly utilized in neuroscience as a highly efficient and reproducible
30 method for the visualization and analysis of neuronal morphology. The use of lipophilic
31 carbocyanine dyes, combined with particle-mediated biolistic delivery, allows for the non-toxic
32 fluorescent labeling of multiple neurons, including their dendritic arbors and spines, in both
33 living and fixed tissue. Since first described, this novel labeling method has been modified and
34 adapted to fit a variety of research goals and laboratory settings. Diolistic labeling has
35 traditionally relied on the use of a commercially available, hand-held gene gun for the
36 propulsion of coated micro-particles into tissue sections. Recently, laboratory-built biolistic
37 devices have been developed and allow for the increased availability and customization of this
38 method. Here, we discuss one such custom biolistic device and provide a detailed protocol for
39 its use in diolistic labeling. In addition to decreasing the associated costs, the laboratory-built
40 device also overcomes many of the obstacles normally experienced with traditional diolistics,
41 allowing for reliable and reproducible neuronal labeling. The versatility of this method allows
42 for its adaptation to a variety of laboratory settings and neuroscience-related research goals.
43
44 **INTRODUCTION:**

45 Three-dimensional morphological reconstructions of individual neurons and their dendritic
46 arbors have served as the bases for analyzing the structure-function relationships within the
47 nervous system[1-3]. For over a century, the main method for these studies consisted of various
48 modifications of the famed Golgi staining procedure[4], which has proved invaluable in
49 developing our modern understanding of the nervous system[1,2]. However, this method and the
50 various modifications of the silver impregnation of a percentage of the neuronal population are
51 not without drawbacks. In particular, the small population of stained cells, while an advantage
52 in morphological studies, elicits the lingering concern of selection bias[2,5]. The Golgi method also
53 suffers from a limited compatibility with modern immunolabeling techniques and confocal
54 microscopy. Additionally, data analysis following the Golgi method of staining may be
55 negatively influenced by inconsistent neuronal impregnation, sectioning artifacts, and the
56 overlapping of fine dendritic processes that leads to an indistinguishable morphology.
57
58 More recently, the use of neuronal transfection and electroporation methods have allowed for
59 neuronal morphological labeling that circumvent the limitations experienced with Golgi
60 staining[6,7]. While the administration of dye into individual cells through microinjection using
61 intracellular or patch pipettes generates excellent single-cell labeling, the technique is
62 technically demanding and may be vulnerable to sampling bias[6,8-10]. Neuronal transfection
63 relies on the introduction of DNA constructs into target cells and tissues through a variety of
64 methods. One such method of DNA transfer is known as "Biolistic" delivery, in which a gene gun
65 utilizes a pressurized release of gas to propel DNA-coated micro-particles into tissue, crossing
66 the plasma membrane to target the cells[3]. This method does not require the same level of
67 technical expertise needed for conventional intracellular injections[11]. Furthermore, the random
68 sampling used in biolistic delivery is ideal for full-scale quantitative analysis. However, the
69 reliance on DNA transfection for fluorescence expression limits the method to use on living
70 tissues in prepared cultures.
71
72 A more recent advancement from the biolistic approach to the morphological labeling of live or
73 fixed neurons was first reported by Gan *et al.* in 2000[12] and is known as diolistic labeling.
74 Diolistic labeling utilizes the lipophilic fluorescent dye dialkylcarbocyanine (DiI). While DiI has
75 traditionally been used in anterograde and retrograde neuronal tracing, it has also proven to be
76 effective in the fluorescent labeling of the neuronal cell membrane. Utilizing a diolistic
77 approach[13], the ballistic delivery of DiI-coated micro-carriers to fixed or cultured tissue slices
78 allows the DiI to be incorporated into the cellular membrane. This occurs through lateral
79 diffusion in living or fixed tissue, illuminating the cellular morphology. When ballistically
80 delivered to tissue sections through a single pulse of high-purity gas (*i.e.,* helium or nitrogen),
81 individual DiI-coated tungsten particles are embedded in various neurons of the tissue. The
82 micro-carriers pass into the soma of the neuron, while the DiI is captured in the neuronal
83 membrane. Thus, the DiI is allowed to diffuse along the cellular membrane of a single neuron,
84 fluorescently labeling the fine neuronal architecture of dendritic branches and spines[13]. DiI-
85 labeled neurons can be observed through high-resolution imaging, such as confocal or two-
86 photon microscopy, and can be digitally reconstructed in precise detail. The quantification and
87 classification of dendritic branching and dendritic spines can be accomplished with appropriate
88 software packages[14,15].

89

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

113

114 **PROTOCOL:**

115

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

120

121 Note: The following protocol will detail the methods used in the diolistic labeling of fixed brain
122 tissue obtained from rats prepared using transcardial perfusion. Previous studies[16] have utilized
123 similar methods of labeling but without the transcardial perfusion and from various animal
124 species.

125

126 1.   **DiI/Tungsten-Coated Bead Preparation**
127 Note: The DiI (1-1'-Dioctadecyl- 3,3,3',3'-tetra methylindo carbocyanine perchlorate)-coated
128 beads should be prepared at least 72 h in advance of tissue preparation to allow for adequate
129 drying and optimal labeling. Prior reports[1] have shown increased success using CM-DiI when
130 performing additional immunolabeling.

131

132 1.1.   Place a clean glass slide on waxed weigh paper under the fume hood.

1.2.   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.

1.3.   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.

1.4.   Vortex the microfuge tube to ensure that the DiI dissolves completely.

1.5.   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.

1.6.   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.

1.7.   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.

1.8.   Sonicate the DiI/PVP solution for 10 min in a water bath at room temperature.

2.   **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.

2.1.   Immediately following sonication, vortex the DiI/PVP solution vigorously for 10-20 s.

2.2.   Pull 100 μL of the DiI/PVP solution from the middle of the conical tube into a mounted pipette tip cartridge.

2.3.   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.

177

<mark>2.4. 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.</mark>

<mark>2.5. 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).</mark>

<mark>2.6. 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.</mark>

<mark>2.7. 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.</mark>

3. **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.

3.1. Anesthetize the animal and confirm deep anesthesia through an IACUC-approved method. Open the abdominal and thoracic walls, exposing the peritoneal and thoracic cavities.

3.2. 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.

3.3. 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.

3.4. 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.

3.5. Postfix the intact brain for 2 h in 1.5% paraformaldehyde.

3.6. Prepare regions of interest or whole-brain specimens under a dissecting scope, taking

221   care to prevent tissue drying.
222

223   3.7.   Prepare a 4% solution of low-melting point agarose in PBS. Heat the agarose until
224   molten with constant stirring to avoid excessive bubble formation.
225

226   3.8.   Pour the molten agarose into an appropriately sized histology embedding mold for the
227   brain tissue and keep it on a 40 °C warming plate. Orient the brain tissue in the desired plane
228   for sectioning and place it in the molten agarose. Remove the agarose from the heat and allow
229   it to cool to room temperature before handling.
230

231   3.8.1.   Avoid placing it in a refrigerated location to speed cooling, as this will negatively affect
232   the embedding and may cause the agarose to break away from the tissue during sectioning.
233

234   3.9.   Remove the cooled block from the mold and trim the excess agarose from the tissue,
235   making sure that all areas of the brain tissue are surrounded by at least 5 mm of agarose.
236

237   3.10.   Secure the tissue block to a mounting block using a cyanoacrylate glue and allow it to
238   dry for a few minutes.
239

240   3.11.   While drying, prepare culture plates by filling each well with 2 mL of PBS.
241

242   3.12.   Place the mounting block onto the vibratome stage and secure it. Lower the stage
243   completely. Attach a clean blade, free of any preserving oils.
244

245   3.13.   Fill the vibratome chamber with cold 25 mM PBS to the specified level so that the blade
246   is covered.
247

248   Note: Only PBS should be use to fill the vibratome well. Lower concentrations or pure water will
249   cause the tissue to swell.
250

251   3.14.   Trim the block in 300-µm increments until the desired section depth is reached.
252

253   3.15.   Vibratome-section the brain into 150- to 300-µm serial sections throughout the region
254   of interest. A high amplitude (8.5 Hz) and low speeds (0.5) are recommended for sectioning
255   brain tissue but should be tested on the vibratome in each laboratory.
256

257   3.16.   Using a paintbrush, remove the sliced tissues from the PBS vibratome bath and transfer
258   them into the tissue culture dishes containing PBS. Store them until the DiI labeling is to be
259   performed.
260

261   Note: Prior experiments have found that tissues may be stored at 4 °C for up to one week prior
262   to labeling without significant impact on tissue labeling[1,17].
263

264   **4.   Diolistic Cellular Labeling**

265 4.1.   Carefully remove previously prepared DiI/tungsten-coated cartridges from the storage
266 container and load them into individual cartridge holders.

267

268 4.2.   Load each individual cartridge holder into the device and set the gas pressure to 120 psi
269 using the regulator.

270

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

273

274 4.3.   Place the culture plate under the device barrel and carefully pull off the PBS using a
275 pipette. Take care to avoid damage to tissue, and ensure that the tissue lays flat on the bottom
276 of the culture plate. Adjust the position using a paint brush, if needed.

277

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

280

281 4.4.   Place a 3.0-µm microplate filter insert over the tissue and lower the barrel to a set point
282 15 mm above the tissue surface. Avoid handling the filter inserts with ungloved hands, as dirt
283 and oils may clog the filter.

284

285 4.5.   Activate the device using a relay switch that allows a 50-ms burst of gas to pass through
286 the device and cartridge.

287

288 4.6.   After the shot, immediately raise the barrel and remove the filter insert. Return the PBS
289 to the well, being careful not to damage the tissue. Take care to ensure that the tissue surface,
290 vertical at the time of labeling, remains vertical for the remainder of the procedure; this is
291 especially important for thicker tissue sections.

292

293 4.7.   Load a new cartridge into the device and repeat the process (steps 4.1-4.6) for each
294 tissue section in the culture plate.

295

296 4.8.   Once completed, protect the entire culture plate from light and allow it to incubate for
297 24 h at room temperature.

298

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

302

303 4.9.   Post-fix the sections for 1 h in 2 mL of 4% paraformaldehyde; this step has proven to be
304 useful in minimizing "overstaining," or the leaching of dye out of neuronal membranes. Wash
305 briefly with PBS and store in fresh PBS at 4 °C after post-fixation.

306

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

309
310 4.10. Mount tissue slices on coated or charged slides and coverslip with an appropriate
311 antifade agent (5% n-propyl-gallate).
312
313 Note: If time allows for imaging in the 24-72 h post-labeling, wet-mounting the slide in PBS and
314 sealing the edges with nail polish or another sealant provides a cost-effect and time-sensitive
315 mounting method. Wet-mounted tissues should be stored in humid chamber at 4 °C when not
316 undergoing microscopy.
317
318 5.   **Confocal Microscopy and Analysis**
319 5.1.   Image the mounted slides with any confocal microscope equipped with a motorized z-
320 stage and lasers between 543 and 561 nm.
321
322 Note: The use of epifluorescence microscopy equipped with TRITC filters will permit the
323 visualization of the DiI-impregnated neurons and can be an important tool in locating areas of
324 interest. However, the use of epifluorescence microscopy in the quantitative three-dimensional
325 morphological analysis of neurons is discouraged. Confocal microscopy allows for increased
326 resolution in images of DiI-impregnated tissues and for optical sectioning through the cells of
327 interest. Digital reconstructions of the optical sections can be assembled into a three-
328 dimensional model for the analysis of morphology[1]. The representative results presented in this
329 report were obtained using sampling parameters detailed by Staffened and Meisel, 2011[1]. It is
330 recommended that sampling parameters and experimentally/statistically appropriate cell
331 numbers and dendritic segments be established before imaging. There are a variety of
332 commercially available software extensions that can be used for image analysis and
333 morphological reconstructions. These product choices should be based on individual laboratory
334 needs.
335
336 **REPRESENTATIVE RESULTS:**
337 Using the procedure presented here, custom diolistic labeling has been used to characterize
338 alterations in neuronal morphology of the lateral/dentate nucleus of the cerebellum. Here, we
339 show representative labeling results from developing rat pups exposed to abnormally high
340 levels of the serotonergic agonist 5-methyloxytryptamine (5-MT), both pre-and postnatally[18].
341 With the aid of quantitative software, dendritic branching morphology (Figure 2), architecture
342 (Figure 3), and synaptic connections (Figure 4) were analyzed.
343
344 Successful diolistic labeling can be rapidly confirmed through cursory visualization with bright-
345 field and epifluorescent microscopes equipped with red (TRITC) filter (Figure 5). Areas of
346 interest (Figure 5A and B) and potential cells for imaging can be identified (Figures 5C, D, and E),
347 though morphological details are often obscured prior to confocal imaging. Unsuccessful
348 labeling (Figure 6) can be caused by improper bead and cartridge preparation or by storage,
349 and care should be taken during steps 1 and 2 of this protocol. Over-labeling (Figure 6) is
350 commonly caused by the aggregations of coated beads in one area of the cartridge (step 2.6)
351 and can be minimized by using the proper technique or by decreasing the bead concentration
352 in the slurry solution (step 1.7). Following confocal image acquisition, max projection z-stacks

353 can be quickly obtained through the use of the open-source image processing resource, ImageJ
354 (NIH), and used to evaluate the criteria for acceptable labeling (Figure 7).
355
356 **Figure 1: Custom biolistic device.**
357 Key features include a baffled barrel to improve particle delivery, a knob for height adjustment,
358 and a fixed base. The firing mechanism consists of a solenoid valve activated by a relay switch
359 that allows a burst of nitrogen gas to pass through the cartridge and filter assembly and into the
360 tissue specimens.
361
362 **Figure 2: DiI-labeled neuron of the lateral/dentate nucleus of the rat cerebellum.**
363 A Z-projection of DiI-labeled neurons are used to illustrated the dendritic morphological
364 analysis. The inset image illustrates the three-dimensional computer tracing models used for
365 the quantification of the morphology. A morphometric software package was used to contour
366 the dendritic processes of the digitized 2-D images and to develop three-dimensional models.
367
368 **Figure 3: Sholl analysis and a comparison of dendritic branching patterns.**
369 Significant differences in branching morphology were observed in experimental (5-MT) groups
370 compared to control (saline) populations. (**) denotes a significance value of $p \leq 0.001$, (*)
371 denotes a significance value of $p \leq 0.05$. This figure has been modified from Hough and Segal
372 2016[18].
373
374 **Figure 4: Analysis of the total dendritic spine numbers and dendritic spine density.**
375 The mean total number of spines per neuron per group (A)[18] and the mean dendritic spine
376 density per group (B)[18]. Representative DiI-labeled dendritic segments used for analysis of
377 control (C) and experimental (D) groups. (**) denotes a significance value of $p \leq 0.001$, (*)
378 denotes a significance value of $p \leq 0.05$. This figure has been modified from Hough and Segal
379 2016[18].
380
381 **Figure 5: Bright-field and epiflorescence overview of tissue sections and an assessment of**
382 **cytoarchitectural boundaries[18].**
383 Composite image (A) of low-magnification bright-field photomicrographs. The lateral/dentate
384 nucleus of the cerebellum (LN) (B) (solid arrow) can be viewed at the lateral aspect of the deep
385 cerebellar nuclei, surrounded laterally by white matter. The medial aspect of the LN abuts the
386 dorsolateral hump (DL) (dashed arrow) of the anterior interposed (AI) nuclei (dotted arrow),
387 with the superior cerebellar peduncle (arrowhead) extending from the hilus of the LN.
388 Epifluorescence photomicrographs at low (10x) (C), medium (20x) (D), and high (60x) (E)
389 magnifications. A single LN neuron (arrow) (C and D) is illuminated with the TRITC filter and can
390 be further imaged using the confocal microscope. High magnification (E) resolves some
391 dendritic segments that extend beyond the focal plane (arrowhead), illustrating the need for
392 confocal imaging at a specific z-depth. The LN is outlined in white (C). This figure has been
393 modified from Hough and Segal 2016[18].
394
395 **Figure 6: Representative over-labeling.**
396 Composite image (A) of low-magnification bright-field photomicrographs representative of

397　failed (over-)labeling with tungsten microparticles (arrow). DiI staining and over-labeling can be
398　visually appreciated by the presence of the pink (arrowhead) coloration outside of a single cell.
399　The epifluorescence photomicrograph (B) shows the over-labeled areas (arrows), with
400　indistinguishable cellular structure.
401
402　**Figure 7: Retrograde versus anterograde labeling.**
403　A laser scanning confocal photomicrograph z-project of LN neurons, representative of
404　retrograde (A) and anterograde (B) DiI labeling. Note the differential appearance of the cell
405　soma (white arrow) with (A) appearing shadowed and incomplete compared to the soma
406　pictured in (B). Care should be taken to avoid including retrograde-labeled neurons in the
407　quantitative analysis if possible.
408
409　**DISCUSSION:**
410　Here, we demonstrate a method of custom diolistic labeling to quantitatively analyze neuronal
411　morphology and synaptic connectivity. The versatility of this method allows for its adaptation to
412　a variety of laboratory settings and research goals. While the results presented here exhibit its
413　use in rat neural tissue, other studies have used diolistic labeling to investigate diverse species
414　through various neuroscience-related applications[16]. The method is relatively fast, as it takes 1-
415　2 days from tissue fixation to image acquisition and has shown promise in its ability to be
416　combined with other, more classical labeling approaches, such as immunostaining[16,19]. With a
417　laboratory-designed and -built device, researchers can take advantage of low-cost materials,
418　which may allow for the method to be incorporated into budget-conscious investigations.
419　Material availability may also assist with increasing the number of tissue sections labeled, in
420　turn increasing the cell numbers used in the analysis while decreasing the effects of failed
421　labeling on a particular study.
422
423　While diolistic labeling has been shown to be a reliable and reproducible method of dendritic
424　labeling, failures in labeling have been reported. To mitigate these failures, it is important to
425　limit variables in cartridge preparation and storage while outlining clear selection criteria for
426　the inclusion of stained neurons for analysis. Neurons suitable for morphological analysis
427　should: (1) be contained within a region of interest, as defined by cytoarchitectural boundary
428　criteria; (2) demonstrate complete fluorescent labeling of well-defined, tapered dendritic
429　endings; and (3) show primary and secondary dendritic trees with clear branching points. To
430　select individual dendritic segments for imaging and spine analysis, the segment should satisfy
431　the following criteria: (1) the entire dendritic segment, as assessed by tapered, well-defined
432　endings, should fall within the optical depth of the tissue section; (2) dendritic segments should
433　be either parallel or at acute angles to the plane of the section to allow for visualization; and (3)
434　segments should not be obscured by the overlap of other dendritic segments at the same z-
435　depth, which may interfere with the quantification of dendritic spines[18,20]. By setting criteria
436　such as these, the impact of sampling bias and variations in DiI labeling can be minimized.
437
438　The most critical step in this protocol is the preparation of the cartridges. Properly prepared
439　cartridges should only exhibit a light haze of beads adhering to the inner wall of the cartridge.
440　Large aggregations of beads lead to the overstaining of the tissue. Additionally, the cartridges

441	should be adequately stored: protected from light, under desiccation, and at a cool
442	temperature. High temperatures can cause the dye to separate from the tungsten bead, while
443	light will decrease the fluorescence of the dye. Harsh handling of the dried cartridges can cause
444	the "dust" of coated beads to fall from the cartridge prior to shooting, resulting in sparse
445	labeling. Current limitations of this method exist in the truncated timetable from labeling to
446	imaging. While an antifade mounting medium can preserve the labeling for extended times,
447	these times pale in comparison to those of other neuronal labeling methods, such as Golgi
448	staining. Experimentation on prolonging tissue labeling by using submersion in antifreeze
449	solutions and storage in sub-zero temperatures is currently underway, with positive preliminary
450	results. Secondary limitations in the method concern the ability to precisely target neuronal
451	populations in a large tissue sample. The device is currently designed to provide a sparse
452	labeling pattern, uniformly spread over a tissue. This approach limits the ability of an
453	investigator to selectively target a population of cells with any accuracy. Future advancements
454	in the firing mechanism and micro-barrel design may facilitate adjustments and refinements in
455	aiming and selection in the future.
456	
457	The diolistic approach to fluorescently label neurons in their entirety has many advantages
458	when compared to more traditional techniques. The rapid nature of the method, the passive
459	diffusion of the dyes, and the compatibility with confocal microscopy provide an unparalleled
460	opportunity to examine features of neuronal cells at a high spatial resolution in a complex,
461	three-dimensional tissue environment. Diolistic labeling should be viewed as a modifiable
462	technique that can be customized to individual research goals and applications.
463	

470	**DISCLOSURES:**
471	The authors have nothing to disclose.
472	

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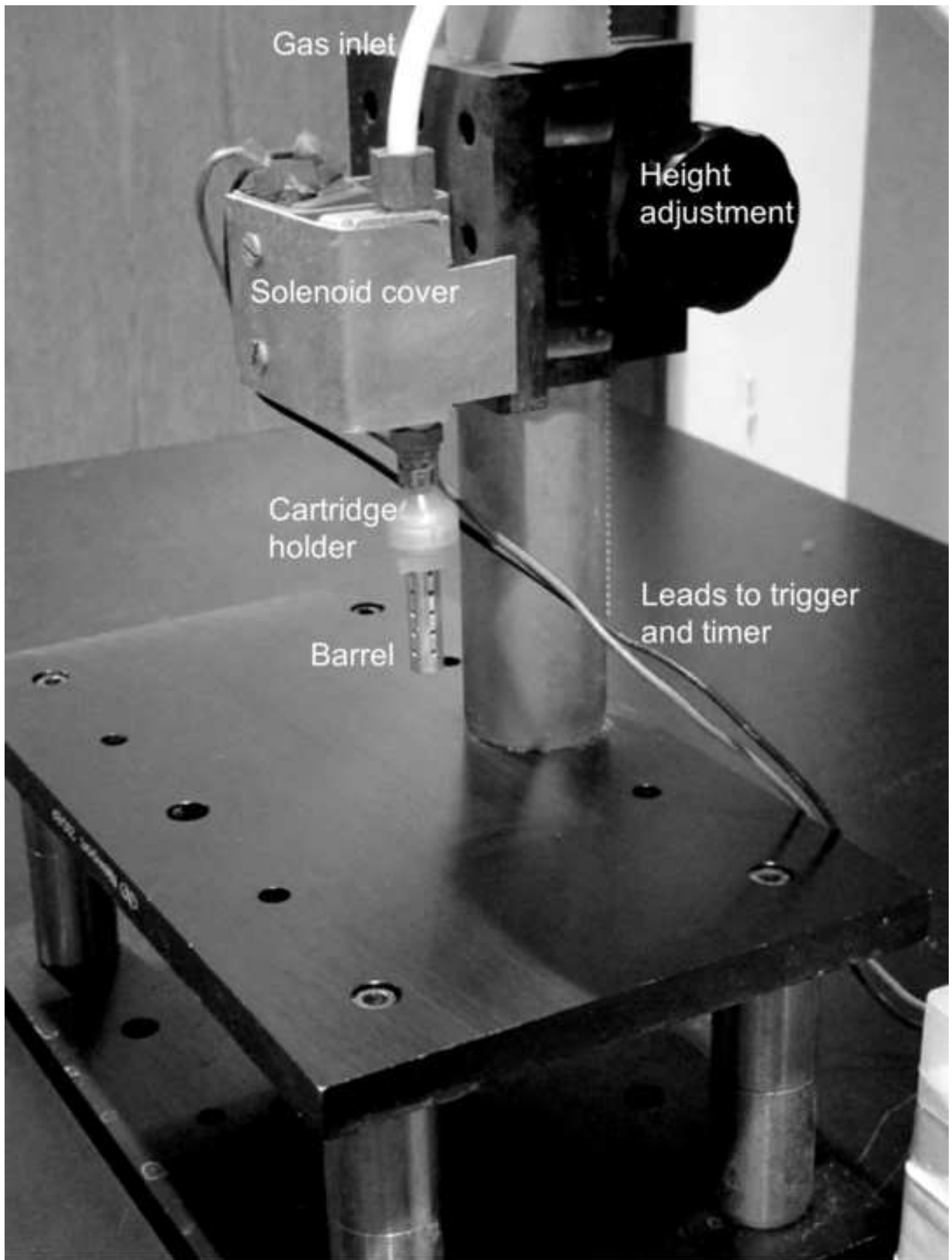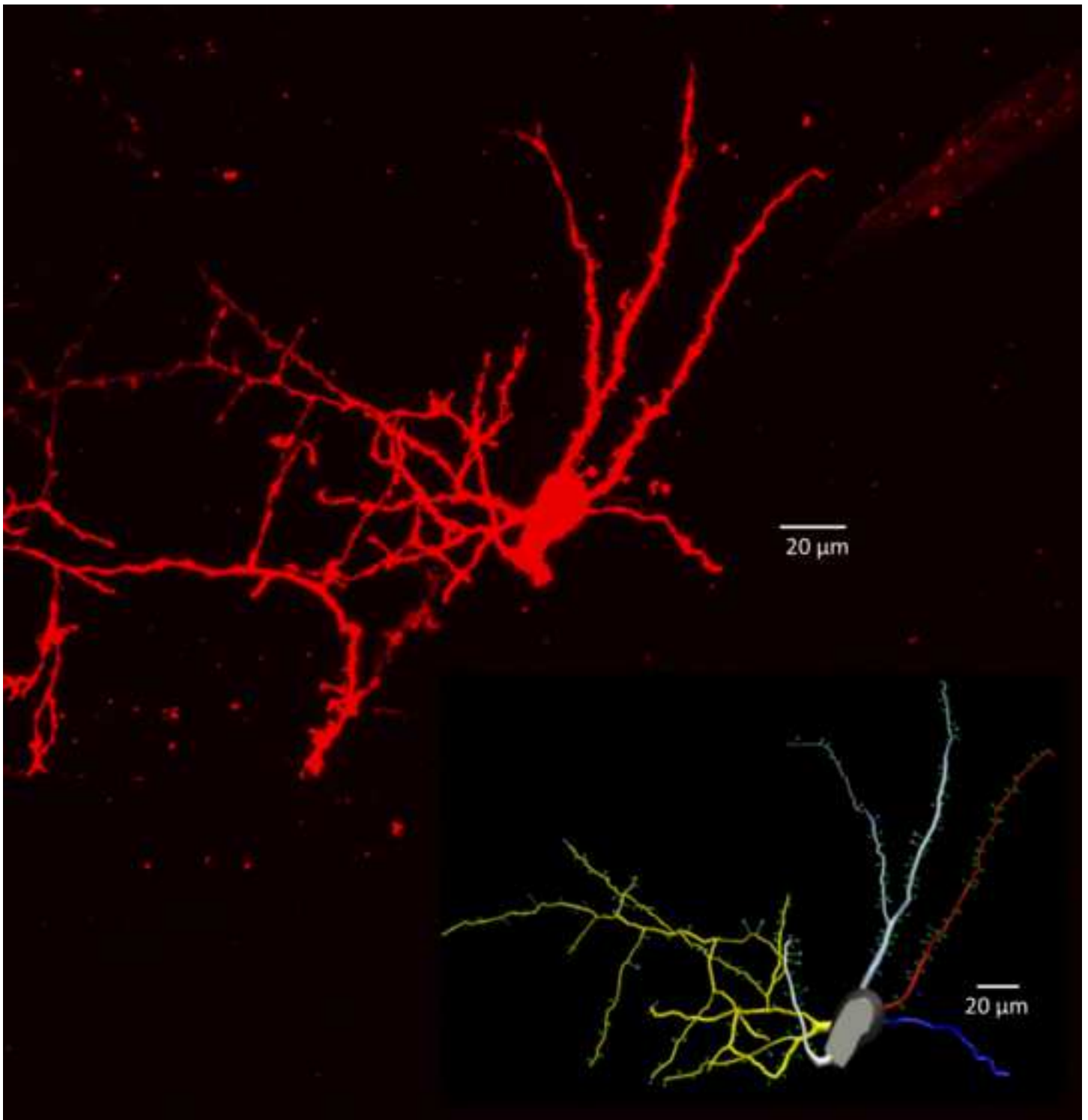524   290 (1998).
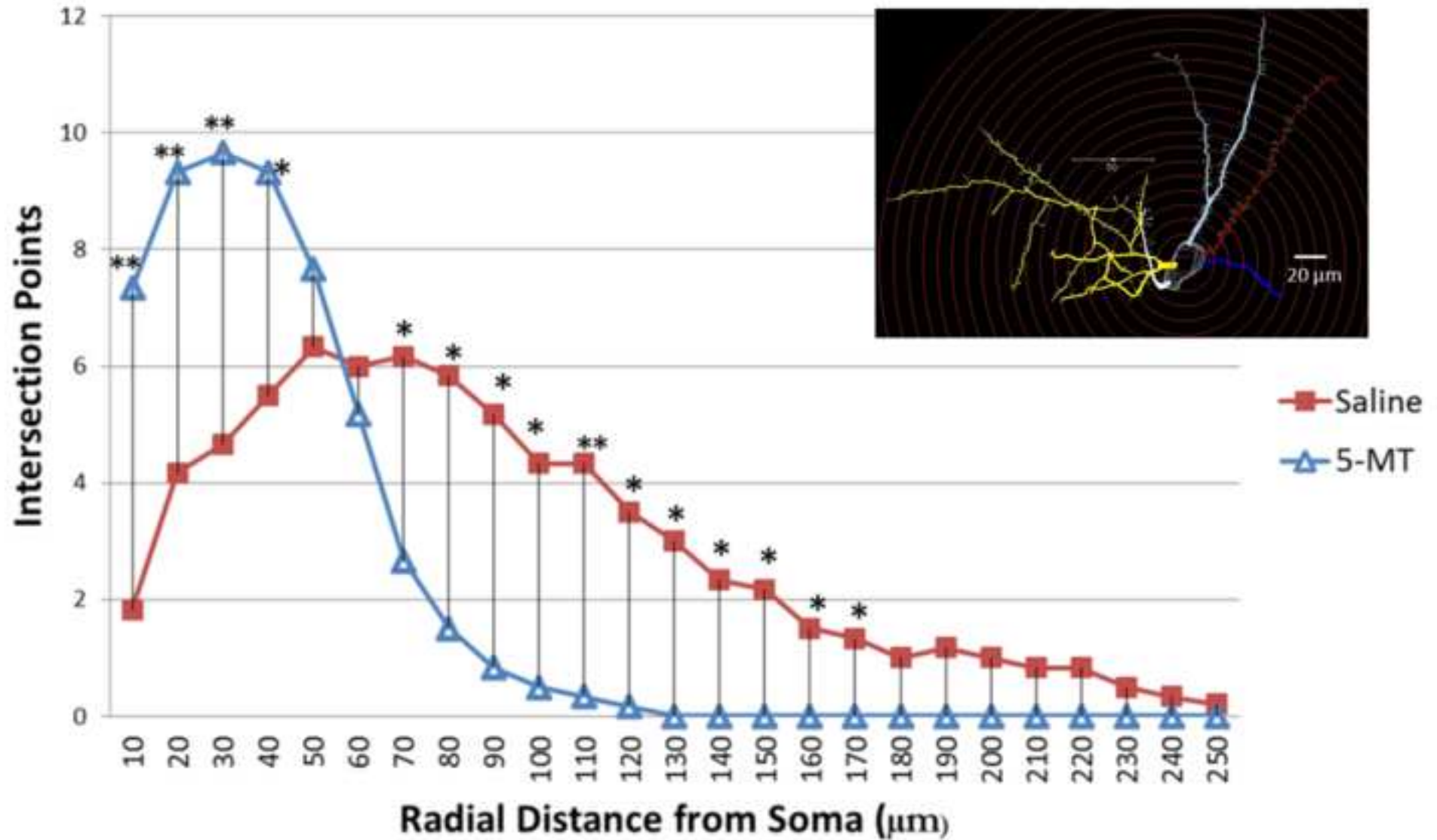
525

Figure 1

Figure 2

Figure 3

Dendritic Intersections
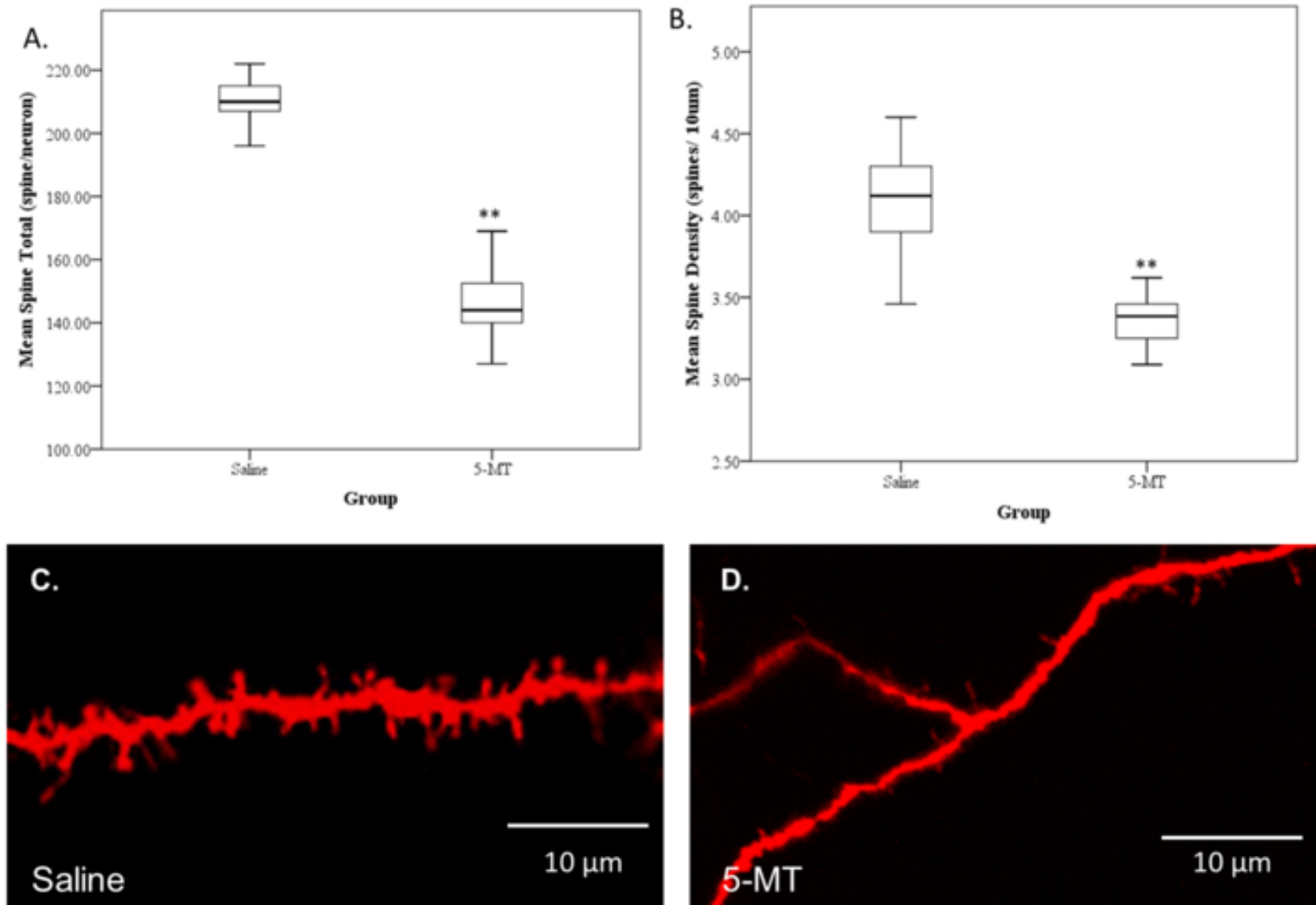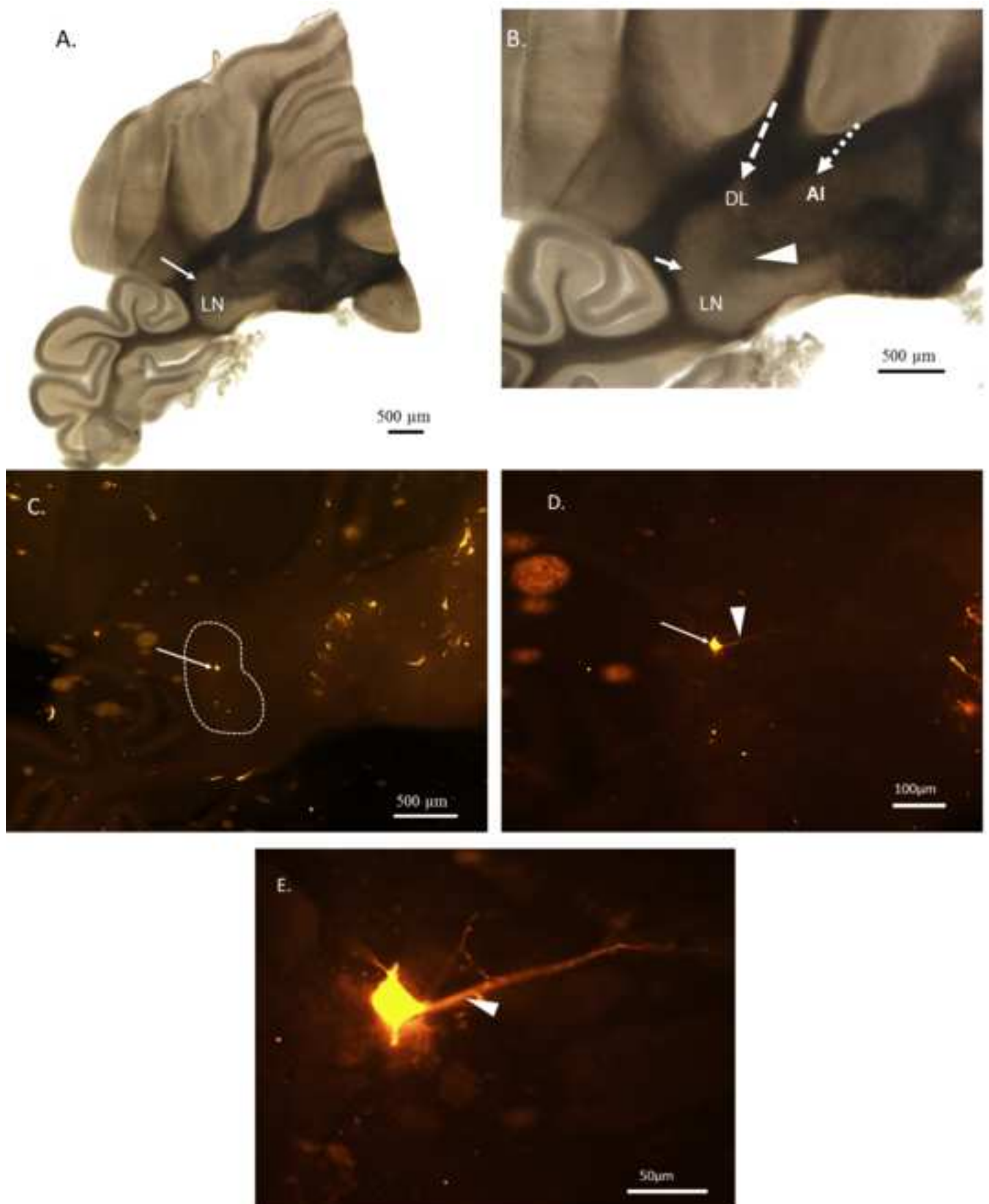
Figure 4

Figure 5

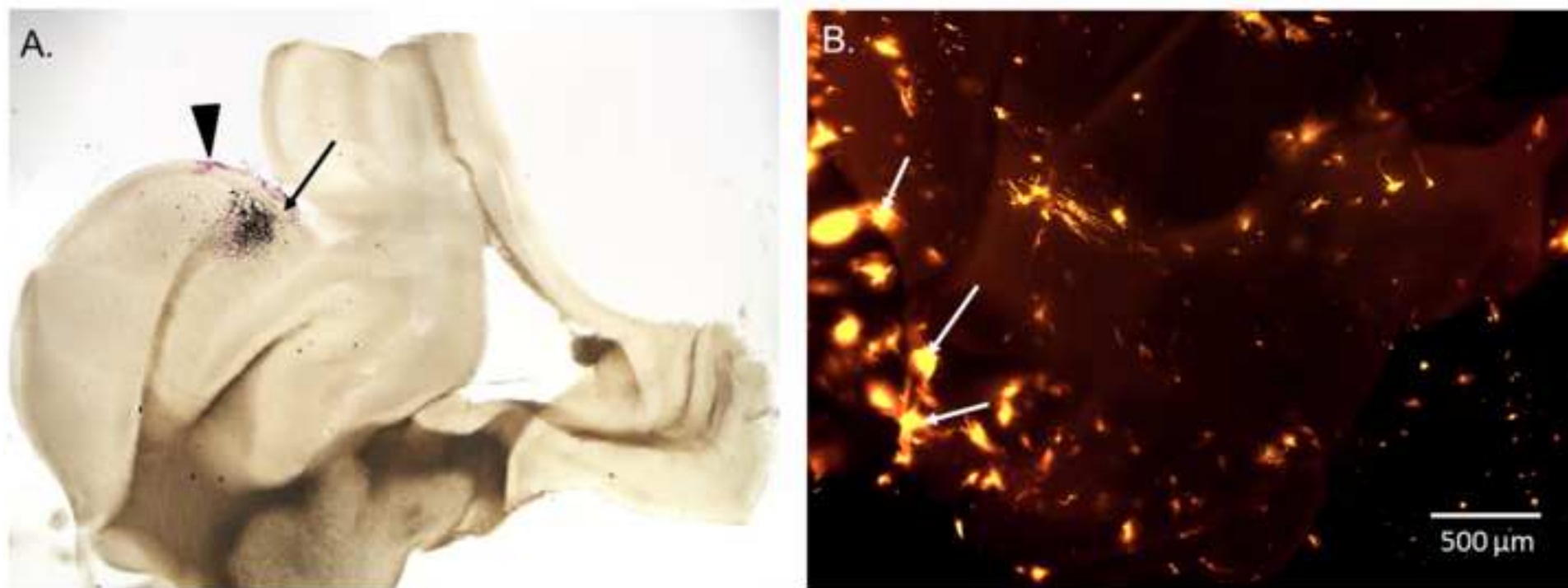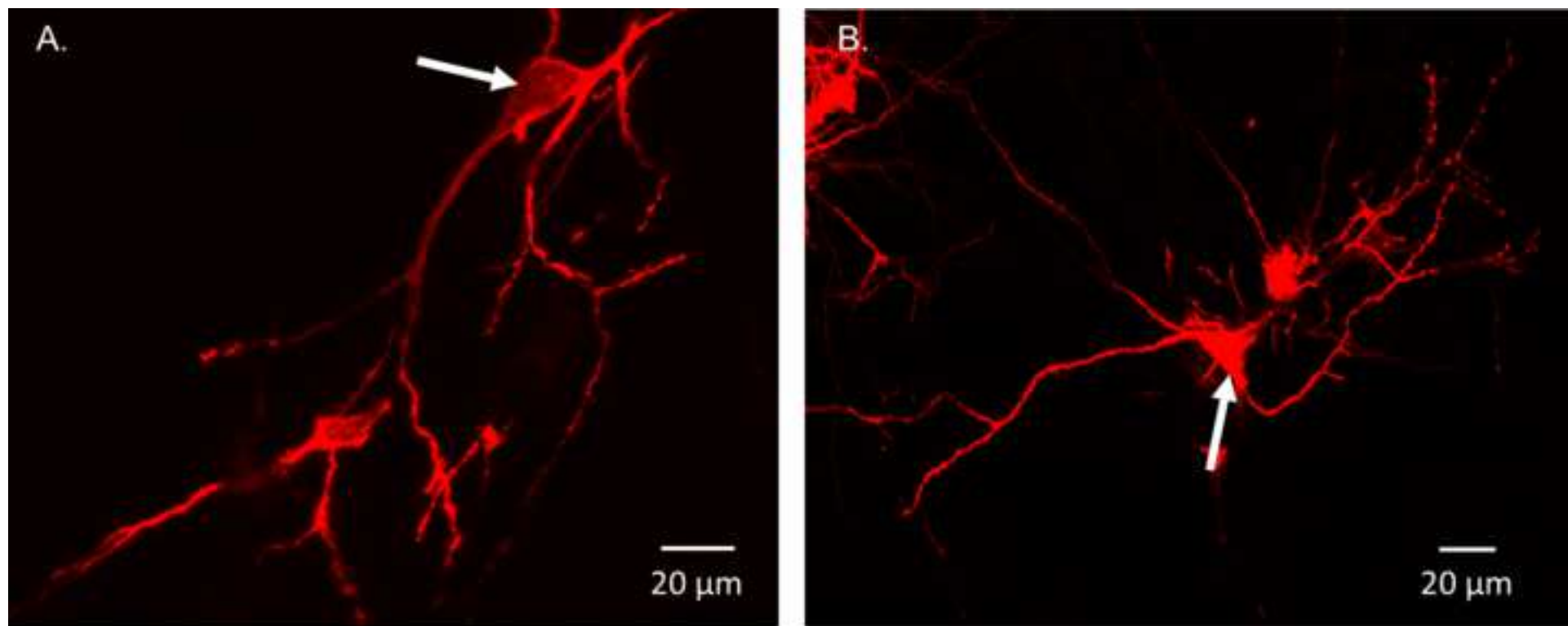Click here to download Figure Figure 5.TIF ⬇

Figure 6

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Figure 7

Name of Reagent/ Equipment

Granular Paraformaldehyde
Sodium Phosphate Monobasic Anhydrous
Sodium Phosphate Dibasic Anhydrous
 Type 1-A, Low EEO Gel Agarose,
Polyvinylpyrrolidone
Dichloromethane (Methylene Chloride)
Yellow Pipet tips 1000pk
Corning Transwell Multiple Well Plate with Permeable Polyester Membrane Inserts
Carbocyanine fluorescent DiI 100mg
ProLong Gold Antifade
1.3-µm Tungsten particles 6G
Millipore Swinnex Filter Holder
Neurolucida Neuron Tracing Software

Company	Catalog Number	Comments/Description
Sigma-Aldrich	P6148-5KG	
Sigma-Aldrich	S2554-500G	
Sigma-Aldrich	71640-1KG	
Sigma Aldrich	A0169-250G	
Sigma Aldrich	77627-100G	
Sigma Aldrich	D65100-1L	
Fisher Scientific	2681151	
Fisher Scientific	07-200-155	
Invitrogen	D-282	
Invitrogen	P36930	
BioRad	165-2269	
EDMmillipore	SX0001300	
MicroBrightField	n/a	

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Author(s): Hough, Lyon H.; Brown, Michael E.

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Rebuttal to editorial and peer review comments

TITLE:
Labeling of Neuronal Morphology using Custom Diolistic Techniques

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Reviewers' comments:
Reviewer #1:
Manuscript Summary:

The JoVE article by Hough and Brown describes the protocol for diolistic labeling of neurons from fixed tissue. It is This article will be a valuable resource for other researchers, particularly because it provides information to improve upon the existing equipment so that it is adapted for this specific application. The BioRad gene gun was not intended for diolistic labeling, and the authors explain how their equipment will improve random labeling of neurons. The authors clearly explain the improvements of their system over existing techniques and over Golgi staining. I look forward to watching the video. I think that it will be important to provide visual details for the assembly of the equipment and for the production of the DiI coated bullet cartridges.

Major Concerns:

There are no major concerns.

Minor Concerns:

It will be important to provide sufficient detail in the video to demonstrate how the equipment is assembled and how the bullets are placed in the chamber.

Author response to Reviewer comments:

Thank you for your comments and thorough review of our manuscript. We agree that a properly narrated video will facilitate the understanding of equipment set-up and cartridge preparation. To insure this, the authors have…

Rebuttal to editorial and peer review comments

Reviewer #2:
Manuscript Summary:
In this paper "Labeling of Neuronal Morphology using Custom Diolistic Techniques" Lyon Hough and Michael E. Brown describe a detailed protocol for lipophilic dye delivery transfection (diolistics) using a custom built device. The main purpose of this paper is to present a "low-cost" diolistic protocol with high reliability and reproducibility. The efficacy of this method is supported by referring to recently published work "Effects of developmental hyperserotonemia on the morphology of rat dentate nuclear neurons".

Major Concerns:
1.) Although the authors tackled in principle a very interesting technical issue for neuroscience, there are a number of excellent reviews already available the same very topic.
2.) The major challenge of this MS is the description of how to utilize an alternative gene-gun apparatus that however has not been developed by these authors and that has already been described by the original authors (Bridgman et al).
3.) However, in this MS there is no sufficient technical description for building up such device. Moreover, another interesting aspect is the use of pipettes tips as cartridges, however also in this case no sufficient description was given : "Particle carriers (cartridges) are supported by a 108 plastic ring that fits inside a modified Millipore filter holder (cartridge holder)12". How should the modified millipore filter be modified in order to obtain a cartridge holder?
4.) The description of neuronal imaging after labelling is very well reported but also not novel. Maybe the authors could have focused their attention to something new such as for instances (as they mention in the MS) the long-time preservation of DiI fluorescence, something that the community is looking for. Or, combining immunofluorescence for synaptic proteins with DiOlistic.
5.) Moreover, although the protocol steps are clearly explained in "protocol section", the reproducibility of this methodology might be hampered by inaccurate description of reagents and materials. In fact, some information are missing (i.e. 3.0 um filters, methylene chloride, pipette tips) and should be added in the table of Material/Equipment. Moreover, it is reasonable that pipette tips retention index might affect loading of DiI-/PVP in cartridge and then compromise Diolistic labeling. In the "protocol section" it is unclear if aldehyde are dissolved in PBS and, differently from the above mentioned paper PBS 25mM is used for all procedures.
6.) Finally, in the "discussion section" data obtained with this protocol should be compared to that obtained using standard gene-gun or other methodology (e.g. Golgi staining) in the same regions.

Minor Concerns:
7.) There are several typos throughout the MS (e.g.: Cartilages should be Cartridges)

Author response to Reviewer comments:
1.) The authors recognize the availability of reviews and even an available JoVE video covering the topic of Diolistic labeling. However, the goal of this manuscript is to provide an alternative approach to the methods previously reported. We feel the strength of the discussed method comes from its adaptability to a variety of laboratory settings and research aims, utilizing more readily available equipment than that previously covered. Additionally, previously reported conflicting information concerning reagents, concentrations, and techniques have been clearly addressed in this updated protocol.
2.) The device previously reported by Bridgeman and Brown was developed, built and tested by Michael E. Brown, coauthor of this manuscript. This same device was modified and protocol optimized by Lyon H. Hough (author) for its use in diolistic labeling and published results in

"Effects of developmental hyperserotonemia on the morphology of rat dentate nuclear neurons". Additional devices have been constructed by Hough utilizing the same basic parameters and available equipment.

3.) While the construction of the device was not covered at length in this manuscript, the authors feel sufficient information and description is given in the manuscript, and will be covered in the video, for readers to undertake construction a similar device with available resources. The authors agree that all modifications to products such as the Millipore filter holders and pipet tips need to have sufficient explanation to allow for reproducibility. We feel these modifications can be best described in a narrated video. Therefore we have highlighted sections of the manuscript protocol to be included in the video demonstration which specifically cover these areas.

4.) The authors have elaborated the statement on line 449 to read "Experimentation on prolonging tissue labeling utilizing submersion in antifreeze solutions and storage in sub-zero temperatures is currently underway with positive preliminary results." We feel the novelty of the manuscript comes from its description of the preparation and labeling protocol, and supports pervious descriptions of neuronal labeling. The goal in this section is to demonstrate that a reader can adapt the method presented here and produce neuronal labeling results previously only possible with commercialized equipment and proprietary reagents.

5.) Thank you for the suggestion, microfilter inserts, pipette tips, and methylene chloride used in this procedure have been added to the included equipment table. The authors have added the statement "Low-retention pipette tips should not be used in this protocol as it may interfere with particle adherence to the inner wall of the tip. The protocol outlined here has utilized standard polypropylene research grade pipette tips (Fisher Scientific) throughout testing." To line 159. The statement "prepared with 25mM PBS" has been added to line 192.

6.) While the authors agree that the comparison between Golgi, standard Diolistic, and our customized method would be an interesting prospect, we find that to be outside the goals of this particular work. The goal of this manuscript is to inform the reader of a reproducible protocol which has been adapted from previous works and to provide representative results which can be expected if said protocol is followed. The authors feel the representative e results and previously published works provide sufficient evidence as to the efficacy of the method.

7.) Thank you for your critical review of our manuscript. Typographical errors have been addressed.

27 December 2016

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Dear JoVE Editorial Board,
        All editorial suggestions have been addressed.

Thank you

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Editor comments:

1. Before we can accept the manuscript, some of the language has to be revised to be original. There are a few lines in the Introduction (second and third paragraph) and Section 5 of the protocol need to be revised. Please see the attached Ithenticate report. We cannot accept previously published text as it poses copyright issues in publication. As section 5 is not highlighted for filming, one option would be to drastically reduce this section and cite a reference instead.

Author response: The sections have been reworded so as to not infringe on copywrite restrictions. Citations continue in the document as needed.

2. Please revise the numbering of the references in order of appearance. Currently, the first cited reference in number 2 in the Introduction. Please do not use references in the short and long abstract.

Author response: References have been reordered as necessary

3. Is it Diolistic, diolistic, or DiOlistic? Also, there continues to be many typos throughout the manuscript. "while and advantage"; "to the well carful not the damage the tissue" Please copy-edit the manuscript.

Author response: The term has been published several different ways in the past. The authors believe the use of diolistic labeling to be the most effective as it closely corresponds to the term biolistic. The term does not warrant capitalization on any grammatical terms and should not be used in quotation as it is no longer deemed a novel method named only by the current author. We have used the un-capitalized diolistic throughout the manuscript except where it begins a sentence.

4. Please remove the commercial branding in the manuscript. Please use the Materials Table to identify the materials and vendor instead: Fisher Scientific, Millipore, Neurolucida, etc.

Author response: commercial branding has been removed. We have elected to leave ImageJ (NIH) in the paper due to its open access nature.

5. For Figure 5, please describe Panels D and E. Is there a typo here? C is 10X, D is 20X, and E is 60X? This is not explained
6.

Author response: There was a typo found here which has been removed. The authors find the addition to clarify any confusion.

Figure usage agreement)
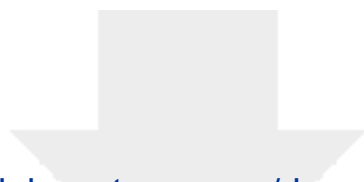
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