Protocol

Coating Particles with Carbocyanine Dyes

Josh L. Morgan and Daniel Kerschensteiner

Imaging and reconstruction of developing neurons require cells that are labeled in a way that distinguishes them from their neighbors. This can be achieved with ballistic labeling, which refers to the delivery of a cell label by means of carrier particles (tungsten or gold) propelled from a pressurized gun. Ballistic delivery can reach many dispersed cells in one shot and can deploy a wide variety of cell markers to neurons in diverse preparations. The three most commonly used types of ballistic labels are carbocyanine dyes, dextran-conjugated fluorescent markers, and DNA plasmids. The primary advantage of ballistic labeling is that multiple dispersed cells can be labeled quickly in live or fixed tissue. This article describes a protocol for coating tungsten particles (~1 μm in diameter) with carbocyanine dyes, which are widely used to label neurons in tissue and neural cells in suspension. These dyes are lipophilic and highly fluorescent within lipid bilayers. Because tissue damage worsens with the increasing pressure required for deeper bullet penetration, ballistic labeling of neurons is most effective when the target cells are near the surface of the preparation. This protocol was developed for labeling ganglion cells in retinal flat mounts.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

Reagents

Carbocyanine dyes (Invitrogen)

The dyes used in this protocol are octadecyl (C18) indocarbocyanines (DiI and DiD) and oxacarbocyanine (DiO).

Ethanol (70% and 100%)

Methylene chloride (Sigma-Aldrich)

Polyvinylpyrrolidone (PVP) (20 mg/mL in ethanol)

Tungsten particles (1.0–1.7 μm diameter) (Bio-Rad Laboratories)

Equipment

Conical tubes (15 mL) (Falcon)

Desiccant pellets

Glass slides

Guillotine (Bio-Rad Laboratories)

Adapted from *Imaging in Developmental Biology* (ed. Sharpe and Wong). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Microcentrifuge tubes (1.5 mL)
Nitrogen gas (ultrapure)
Razor blades
Sonicator
Syringe (5 or 10 mL; fitted with flexible connector to fit Tefzel tube; see Fig. 1D)
Tefzel tubing (Bio-Rad Laboratories)
Tubing preparation station (Bio-Rad Laboratories) (optional; see Step 3)
Vial (e.g., screw-cap scintillation vial)
Weighing paper (wax-coated)

METHOD

1. Dilute PVP in 100% ethanol to give a final concentration of 0.01 mg/mL.
2. Using a syringe, draw the PVP solution into 75 cm of Tefzel tubing.
3. Allow the tubing to rest for 2 min on a benchtop before withdrawing the solution from the tubing.

   A tubing preparation station designed as part of the Helios Gene Gun system (Bio-Rad Laboratories) is convenient for wetting the tubing, as well as drying and coating it.
4. Dry the tubing by purging it with ultrapure nitrogen gas while completing the remaining portion of the dye preparation.

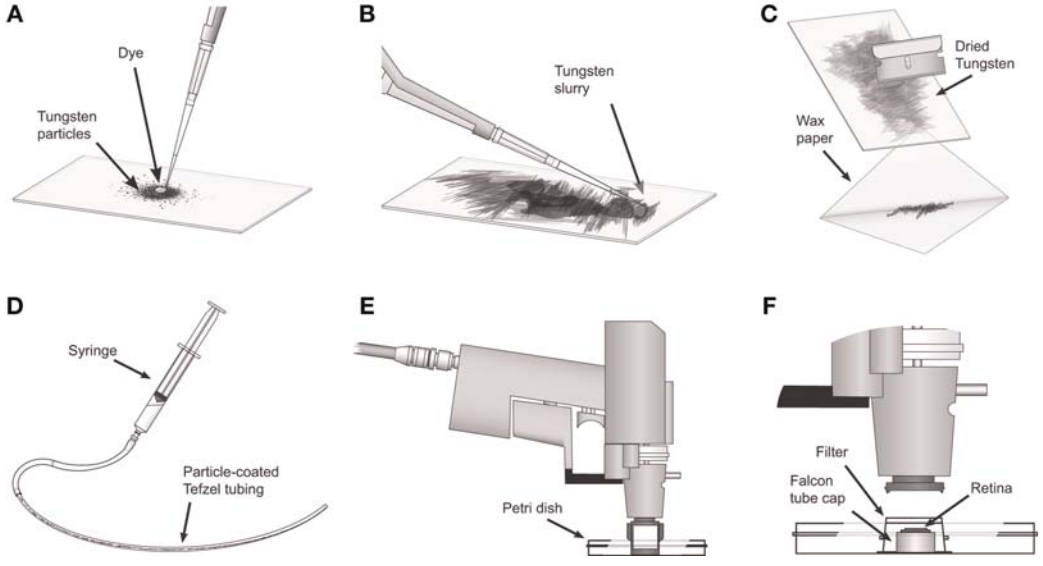

FIGURE 1. Particle coating and shooting tissue with a gene gun. (*A*) Add dye to tungsten particles on a clean glass slide. (*B*) Mix dye with tungsten, and spread across the slide to create a thin smooth film. (*C*) Gently scrape dried particles onto wax paper. (*D*) Use a 5-mL syringe to load the Tefzel tube with suspended carrier particles and to draw off the fluid once the particles have settled. (*E*) When shooting DNA-coated particles at low pressure, the standard Helios Gene Gun barrel can be used without a filter between the gun and the tissue. (*F*) Shooting particles coated in dye or shooting DNA-coated particles at high pressures requires that a filter be placed between the gun and the tissue. The front spacer of the Helios Gene Gun barrel can be removed to achieve the proper distance between the gun and the filter (~1 cm). The protocol for shooting coated particles into tissue slices is described in **Shooting DNA, Dyes, or Indicators into Tissue Slices Using the Gene Gun** (Morgan and Kerschensteiner 2011).

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5. Place the carbocyanine dye in a 1.5-mL microcentrifuge tube. For DiI, use 2 mg; for DiO, use 4 mg; and for DiD, use 2.5 mg. Dissolve each dye in 200 µL of methylene chloride, and vortex.

   For preparing dye combinations to perform multicolored (seven-color) shooting, see Gan et al. (2000).

6. Place 100–200 mg of 1.0–1.7-µm tungsten particles on a clean glass slide. Use a different slide for each dye.
7. Add dissolved dye to the tungsten particles, and use the tip of the pipette to mix particles and dye while spreading them across the surface of the glass slide to form a thin film (Fig. 1A,B).
8. Allow the particles to dry for several minutes.
9. Once the particles are dry, use a clean razor blade to gently scrape the dye-coated particles from the slide, and chop up any clumps that might remain. The result should be a fine powder (Fig. 1C).
10. Use weighing paper to transfer tungsten particles into a 15-mL conical tube.

    Particles coated with different color dyes can be combined at this stage for rainbow labeling of tissue.

11. Add 3–5 mL of distilled H_2O to the powder(s), vortex, and sonicate for 1–2 min.
12. Draw the slurry into the PVP-coated tubing using a 5- or 10-mL syringe fitted with a small piece of silicon tubing that fits over the PVP-coated tubing (Fig. 1D).
13. Allow the particles to settle for 2–3 min, and then carefully draw off the supernatant using the syringe. Do not disturb the tungsten particles.
14. Dry the tubing in a gentle stream (0.2–0.4 L/min) of nitrogen.

    See Troubleshooting.

15. Cut the dried tubing using the guillotine, and collect the cartridges on a large Kimwipe.
16. Gently shake the cartridges in the Kimwipe (holding the ends of the cleaning Kimwipe together). This is important to ensure an even dispersion of the particles in the tubing.
17. Place the cartridges in a capped vial together with a desiccant pellet, and store at room temperature for up to several months.

    Once the particles have been coated and inserted into the tubing that serves as the casing, the cartridges are stable for several months at room temperature, provided they are kept dry. Proceed to the protocol described in ***Shooting DNA, Dyes, or Indicators into Tissue Slices Using the Gene Gun*** *(Morgan and Kerschensteiner 2011) for details on shooting carbocyanine dye into tissue slices using the gene gun.*

TROUBLESHOOTING

Problem (Step 14): Carrier particles fail to stick to Tefzel tubing.

Solution: PVP can be used to increase adhesion. Higher concentrations of PVP can be used to increase particle adhesion during coating, but higher helium pressures may then be required to purge the cartridge (see **Shooting DNA, Dyes, or Indicators into Tissue Slices Using the Gene Gun** [Morgan and Kerschensteiner 2011]).

DISCUSSION

The procedure described here was optimized for labeling ganglion cells in retinal flat mounts using a Helios Gene Gun (Bio-Rad Laboratories). The large surface area (~35 mm^2) of this preparation and the superficial location of ganglion cell somata (<20 µm deep) make them an ideal target for ballistic labeling. This coating protocol can also be adapted to other dyes that are soluble in methylene chloride (Gan et al. 2000; O'Brien and Lummis 2004, 2006, 2007).

Carbocyanine dyes are bright lipophilic dyes that rapidly label cell membranes (Fig. 2A,B). Ballistic labeling of tissue will usually produce a range of labeling intensities and it is important to note that heavy labeling with carbocyanine dyes tends to be toxic to cells even before photoexcitation and

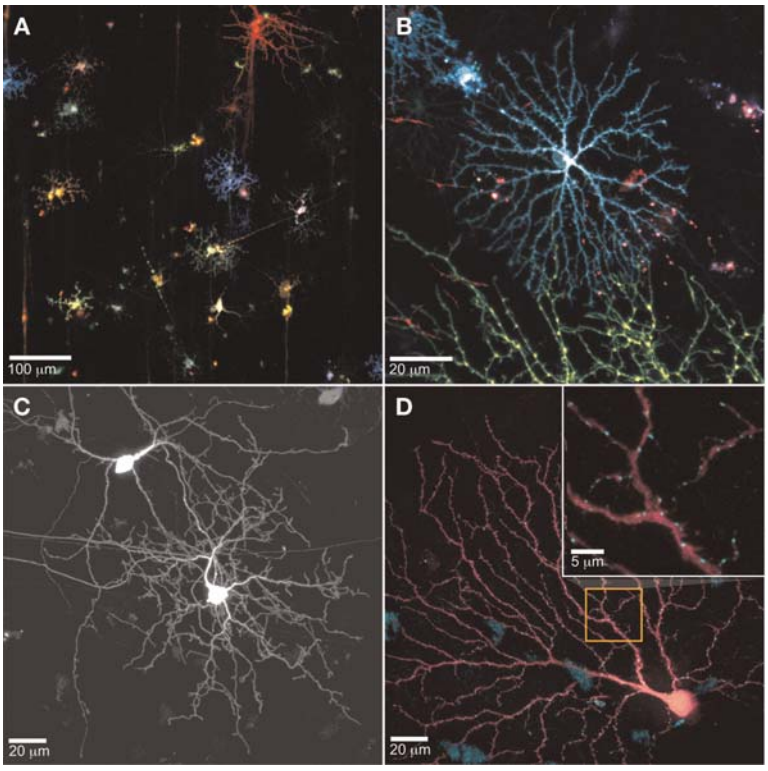

FIGURE 2. Examples of retinal neurons ballistically labeled with different types of molecules. (*A*) Multicolor labeling of cells in the neonatal mouse retinal ganglion cell layer using particles coated with different combinations of carbocyanine dyes (DiI, DiO, DiD) (see Gan et al. 2000). (*B*) Spectrally distinct membrane labeling of cells with multicolored carbocyanine dye. (*C*) Cytosolic labeling of two neonatal mouse retinal cells with dextran-conjugated Fluoro-Ruby. (*D*) A neonatal mouse retinal ganglion cell expressing cytosolic td-Tomato (magenta) and yellow fluorescent protein-tagged (YFP-tagged) postsynaptic density 95 (PSD95, cyan puncta). PSD95 is a scaffolding protein found at glutamatergic postsynaptic sites. Expression was driven by coating particles with two plasmids, *CMV*: td-Tomato and *CMV*: PSD95-YFP (Morgan et al. 2008). *Inset* shows the region within the orange box at higher magnification. (*A,B,* Reprinted, with permission, from Lohmann et al. 2005.)

possible phototoxic effects. In contrast, dextran-conjugated dyes such as Fluoro-Ruby (Fig. 2C) and Oregon Green rapidly fill the cytosol of a cell and are generally less toxic than carbocyanine dyes (Stacy and Wong 2003). However, labeling cells with DNA-coated particles provides, by far, the greatest flexibility, because genetic constructs can be made from fluorescent proteins that have been fused to other proteins to specifically label particular subcellular structures (Fig. 2D) (Lo et al. 1994; Wong et al. 2000; Morgan et al. 2008). The range of applications for ballistic DNA transfection of cells (biolistics) is limited primarily by the relatively long incubation time (6–24 h in retinal ganglion cells) required for sufficient protein expression.

Tissue that has been labeled by ballistic delivery can be imaged using standard imaging methods. The multicolor labeling of superficially located cells lends itself to confocal imaging, although most fluorescent labels can also be efficiently (two-photon) excited with an infrared laser. A sufficiently dense layer of carrier particles will reduce image quality, but the particle density at which image quality is significantly reduced is generally greater than the density at which tissue health is compromised.

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