Video Article

Localized RNAi and Ectopic Gene Expression in the Medicinal Leech

Orit Shefi[1], Claire Simonnet[2], Alex Groisman[2], Eduardo R Macagno[1]

[1]Division of Biological Sciences, University of California San Diego - UCSD

[2]Department of Physics, University of California San Diego - UCSD

Correspondence to: Orit Shefi at oshefi@ucsd.edu

URL: http://www.jove.com/video/697
DOI: doi:10.3791/697

Keywords: Neuroscience, Issue 14, leech, netrin, axon guidance, development, mechanosensory neurons, gene gun, RNAi

Date Published: 4/17/2008

Citation: Shefi, O., Simonnet, C., Groisman, A., Macagno, E.R. Localized RNAi and Ectopic Gene Expression in the Medicinal Leech. *J. Vis. Exp.* (14), e697, doi:10.3791/697 (2008).

Abstract

In this video, we show the use of a pneumatic capillary gun for the accurate biolistic delivery of reagents into live tissue. We use the procedure to perturb gene expression patterns in selected segments of leech embryos, leaving the untreated segments as internal controls.

The pneumatic capillary gun can be used to reach internal layers of cells at early stages of development without opening the specimen. As a method for localized introduction of substances into living tissues, the biolistic delivery with the gun has several advantages: it is fast, contact-free and non-destructive. In addition, a single capillary gun can be used for independent delivery of different substances. The delivery region can have lateral dimensions of ~50-150 μm and extends over ~15 μm around the mean penetration depth, which is adjustable between 0 and 50 μm. This delivery has the advantage of being able to target a limited number of cells in a selected location intermediate between single cell knock down by microinjection and systemic knockdown through extracellular injections or by means of genetic approaches.

For knocking down or knocking in the expression of the axon guidance molecule Netrin, which is naturally expressed by some central neurons and in the ventral body wall, but not the dorsal domain, we deliver molecules of dsRNA or plasmid-DNA into the body wall and central ganglia. This procedure includes the following steps: (i) preparation of the experimental setup for a specific assay (adjusting the accelerating pressure), (ii) coating the particles with molecules of dsRNA or DNA, (iii) loading the coated particles into the gun, up to two reagents in one assay, (iv) preparing the animals for the particle delivery, (v) delivery of coated particles into the target tissue (body wall or ganglia), and (vi) processing the embryos (immunostaining, immunohistochemistry and neuronal labeling) to visualize the results, usually 2 to 3 days after the delivery.

When the particles were coated with netrin dsRNA, they caused clearly visible knock-down of netrin expression that only occurred in cells containing particles (usually, 1-2 particles per cell). Particles coated with a plasmid encoding EGFP induced fluorescence in neuronal cells when they stopped in their nuclei.

Video Link

The video component of this article can be found at http://www.jove.com/video/697/

Protocol

Particles can be coated with different reagents, such as dsRNA molecules, DNA plasmids or dyes. The type and diameter of particles are chosen according to the reagent and the depth of the target cells in the tissue: larger particles penetrate further, but may cause some damage to the tissue.

1. Preparation of dsRNA coated particles

1. Place 100% isopropanol on ice.
2. Resuspend 5 mg gold particles (S1600ri, Seashell Technology, LLC; average diameter 1.6 μm) in 100 ml binding buffer. When solution is ready, continue to the next step.
3. Dilute the particle solution by adding 100 μl of binding buffer, vortex briefly and sonicate for 1-2 min to avoid clumping.
4. Add 5-10μg of dsRNA/siRNA (dsRNAs/ siRNA should be dissolved in water to a concentration of ~1μg/μl).
5. Vortex and leave at room temperature for 2 min. These concentration and time interval result in siRNA saturated gold particles.
6. Pellet the siRNA/gold particle complexes by centrifugation at ~2,500 rpm in a bench top centrifuge for ~ 15 sec.
7. Remove the supernatant and gently add 750 ml cold isopropanol with minimal disruption of the pellet. Spin briefly and remove the supernatant.
8. Resuspend the gold particles in 100 μl cold 100% isopropanol, then sonicate briefly in a bath sonicator (2-3 pulses) to break up particle agglomerates. Last, pour the suspended particles onto a glass slide and allow to dry at room temperature.

2. Preparing the experimental setup for a specific assay

1. Each gun has a different nozzle aperture diameter, ranging from a minimum of ~50µm up to ~3mm. The specific to be used for the gun is therefore chosen according to the area of tissue to be targeted.
2. The He pressure is adjusted for the depth in the tissue of the target cells, generally up to ~100µm (higher pressures can deliver the particles deeper). The distance between the embryo and the tip of the nozzle also affects the penetration depth, as gold particles loose momentum in air.

3. Loading the gun with the pre-coated particles

Particles should be loaded as a dry powder into the tygon tubing particle injection lines connected to the manifold. This tubing, which should be kept in a dry atmosphere at 4°C when not in use, can be re-used in additional experiments with the same reagents. Our experimental setup can deliver up to two different reagents per assay through separate ports in the manifold.

1. Scrape the dried coated particles from the glass slides using the edge of a glass cover slip or a razor blade before loading them into the tygon tubing line. This tygon tubing will be specific only to this reagent to avoid cross contamination.
2. As you load the particles, bend the tubing into a U shape close to the connector, to keep the particles from spreading along the whole tubing and concentrated close to the connector. Pick up the particles with a small spatula or a piece of folded weigh paper, and load about half of them into the tubing for each series of embryos. Tap the tubing during the loading step to spread the particles evenly.

4. Animal Preparation

Before and after the gold particle delivery, the embryos are kept in sterile artificial pond water at room temperature.

1. Place the embryos, ventral side up, in a groove carved into a flat, 5mm-thick piece of silicone rubber (Sylgard 184, Dow Corning, Midland, MI) and anaesthetize them in a solution of 8% ethanol in sterile artificial pond water (younger embryos at stages E6-E8 can remain in artificial pond water with no ethanol throughout the experiment).
2. Immediately before shooting the particles, lower the bath level to uncover the ventral surface of the embryos by removing part of the solution. Place a piece of tissue paper with a small hole cut in the middle on top of it to stabilize it and to keep its surface from drying.

5. Delivery of coated particles into the target tissue

One load of particles can typically be used for up to ten shots, with a single shot usually delivering on the order of a few hundred particles.

1. Generate a shot by opening one of the valves for 0.3 s, causing the injection of a bolus of particles from the corresponding tubing line into the gun.
2. Tap the tubing gently between the shots to dislodge particles from the tubing wall and thus facilitate their injection into the He stream of the gun.
3. To cover the area of tissue of interest, multiple shots may be required.
4. After the particle delivery, place the embryos back into artificial pond water, preferably one embryo per well in a multi-well plate.

6. Immunostaining and neuronal labeling

Keep the embryos at room temperature and in darkness for 1 to 3 days after the particle delivery, until RNAi (or ectopic expression) is achieved. We then carry out one of the following procedures on the experimental specimens and controls:

1. In situ hybridization to assay for the presence or absence of Netrin mRNA. Digoxigenin-labeled leech netrin riboprobes are hybridized to whole leech embryos.
2. Immunocytochemistry to assay for Netrin protein and other proteins which are unique to neuronal tissue (such as anti acetylated tubulin). The procedure is performed also on whole-mount embryos.
3. Microinjections of lipophilic dyes (DiI and DiO) into single neurons, for dye-fills to label the complete arbors of the neurons of interest (mechanosensory pressure neurons in our case) in treated and control segments. This assay is performed on live embryos (intact or dissected).

Discussion

In this demonstration, we used the pneumatic capillary gun to deliver gold particles into cells in small localized volume of a live leech embryo to knock-in and knock-down genes. The target region generally had a diameter of ~150 µm and particles were found ~15 µm around the mean penetration depth, which was adjustable between 0 and 50 µm. When the particles were coated with dsRNA molecules of the axon guidance factor netrin, they caused clearly visible knock-down of netrin expression that only occurred in cells containing particles. Particles coated with a plasmid encoding GFP induced fluorescence in neuronal cells when they stopped in their nuclei.

We have shown you how the pneumatic capillary gun gives the technique of biolistic delivery a new capability, to target microscopic regions of a tissue confined in three dimensions and targeted with high precision, without detectable damage to the tissue.

Acknowledgements

We would like to thank Dr. Michael Baker for his help in generating constructs for gene knock-in and for providing the time-lapse movies of growing sensory projections. We would also like to thank Virginia Vandelinder for technical assistance with the pneumatic gun experiments.

References

1. Baker, M. W. & Macagno, E. R. .RNAi of the receptor tyrosine phosphatase HmLAR2 in a single cell of an intact leech embryo leads to growth-cone collapse. Current Biology 10, 1071-1074 (2000).

2. Baker M.W. and Macagno E.R., Characterization of Hirudo medicinalis DNA Promoters for Targeted Gene Expression. Journal of Neuroscience Methods 156: 145-153 (2006).

3. Gan, W. B. et al. Cellular expression of a leech netrin suggests roles in the formation of longitudinal nerve tracts and in regional innervation of peripheral targets. Journal of Neurobiology 40, 103-115 (1999).

4. Juven-Gershon T., Cheng S. and Kadonaga J.T., Rational design of a super core promoter that enhances gene expression. Nature Methods 3(11): 917-922 (2006).

5. Rinberg, D., Simonnet, C. & Groisman, A. Pneumatic capillary gun for ballistic delivery of microparticles. Applied Physics Letters 87, 014103 (2005).

6. Shefi O., Simonnet C., Baker M.W., Glass J.R. and Macagno E.R., Microtargeted gene silencing and ectopic expression in live embryos using biolistic delivery with a pneumatic capillary gun. Journal of Neuroscience 26: 6119-6123 (2006).

