Chapter 7

Helios® Gene Gun–Mediated Transfection of the Inner Ear Sensory Epithelium

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Abstract

Helios® Gene Gun–mediated transfection is a biolistic method for mechanical delivery of exogenous DNA into cells *in vitro* or *in vivo*. The technique is based on bombardment of a targeted cellular surface by micron- or submicron-sized DNA-coated gold particles that are accelerated by a pressure pulse of compressed helium gas. The main advantage of Helios® Gene Gun–mediated transfections is that it functions well on various cell types, including terminally differentiated cells that are difficult to transfect, such as neurons or inner ear sensory hair cells, and cells in internal cellular layers, such as neurons in organotypic brain slices. The successful delivery of mRNA, siRNA, or DNA of practically any size can be achieved using biolistic transfection. This chapter provides a detailed description and critical evaluation of the methodology used to transfect cDNA expression constructs, including green fluorescent protein (GFP) tagged full-length cDNAs of myosin XVa, whirlin, and β-actin, into cultured inner ear sensory epithelia using the Bio-Rad Helios® Gene Gun.

Key words: Biolistic, transfection, Gene Gun, culture, ear, hair cell, stereocilia, myosin, whirlin, actin, immunofluorescence, GFP.

1. Introduction

Mammalian inner ear sensory epithelia of hearing and balance end-organs (organ of Corti and vestibular epithelia, respectively) are tiny delicate tissues embedded in a bony labyrinth of the temporal bone and are not readily accessible for non-traumatic, non-surgical interventions. Using explanted (cultured) rodent inner ear neurosensory epithelium, one can study the development and function of these sensory cells *(1, 2)* as well as investigate the effects of epitope-tagged cDNA constructs that are transfected into particular cell types of the inner ear of mice and

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rats *(3–6)*. Moreover, using inner ear tissues from deaf mutant mice, researchers can evaluate the effects of exogenous wild-type cDNA on the phenotype of auditory and vestibular hair cells as well as other cell types from a malfunctioning inner ear *(6, 7)*. However, inner ear hair cells cannot be transfected using conventional transfection techniques such as lipofection. In wild-type animals, auditory hair cells are terminally differentiated cells; they exit the cell cycle during embryonic development and lose their ability to divide. Lipofection is a technique designed to deliver genetic material into a cell by means of liposomes, phospholipid-based vesicles that merge with the cell membrane and release their contents into the cell *(8)*. Lipofection can be used to transfect many cell lines but does not work well with terminally differentiated cells. Another method of delivering exogenous DNA uses viruses *(9–11)*. The drawback for the most commonly used virus vectors is that the maximum DNA insert size is limited *(9)*, and there are concerns over the toxicity and immunogenicity of viral DNA delivery systems *(12)*. An alternative is electroporation-mediated transfection, which is based on the application of an electric field pulse that creates transient aqueous pathways in lipid bilayer membranes, allowing polar molecules to pass *(13)*. Electroporation causes a brief increase in membrane permeability after which the membrane quickly reseals. This method is effective with nearly all cell types and species and is used in many applications, including *in vivo* transfection of embryonic mouse brain *(14)* and *in vitro* transfection of immature hair cells from embryonic inner ear explants *(15)*. One disadvantage of this method is the exposure of targeted and non-targeted cells in complex tissues to a potentially damaging current. Excessive cell damage and death were a long-standing concern *(16)* until electroporation devices were improved *(17)*. However, electroporation so far has not been used successfully to transfect structurally developed, nearly mature hair cells from the auditory epithelium of postnatal mice.

Transfection mediated by calcium phosphate precipitation also has a low efficiency for terminally differentiated cells. However, it was shown recently that co-precipitating adenoviral vectors with calcium phosphate increased gene expression and transduction efficiency in mouse dendritic cells, primarily owing to receptor-independent viral uptake. This approach combines the efficiency of adenoviral-mediated endosomal escape and nuclear trafficking with the receptor independence of non-viral gene delivery *(18)*. Improved transfection efficiency was observed also when combining viral or non-viral vectors with paramagnetic nanoparticles and targeted gene delivery by application of a magnetic field. This method of transfection is called magnetofection *(12, 19, 20)*. While magnetofection does not necessarily improve the overall performance of any given standard gene transfer method *in vitro*, its potential lies in rapid and efficient transfection at low vector doses and the possibility of remotely controlled vector targeting *in vivo (21)*. A microinjection method of delivering exogenous DNA into a cell, although precise, is labor intensive. Recently, a fully automated robotic system for microinjection was developed and used in zebrafish embryos *(22)*. Another method that might allow for targeted transfection of cells is an ultrashort (femtosecond), high-intensity, near-infrared laser that can make a tiny, localized transient perforation in the membrane through which plasmid DNA can enter the cell *(23)*. However, this method has not yet been adapted to transfect inner ear sensory epithelia.

The method of DNA delivery using sub-micron-sized particles (microcarriers) that are accelerated to high velocity was developed in the late 1980s by Sanford, Johnston, and colleagues *(24–26)*. This biolistic method was designed to circumvent difficulties in transfecting plant cells whose cell walls present a physical barrier for simple diffusion and/or internalization of transfected material and was subsequently shown to be applicable to mammalian cells *(26, 27)*. In the early 1990s, it was used to deliver exogenous DNA to tissue in a live mouse *(28, 29)*. Since then biolistic devices were modified for particular applications and used *in vitro* to transfect cultured cells and tissues, from yeast to mouse brain slices *(25, 27, 30–32)*, and *in vivo* for intradermal vaccination of human and animals using DNA and mRNA vaccines *(33, 34)*. In the Bio-Rad handheld Helios® Gene Gun delivery system (Bio-Rad Laboratories, Inc., Hercules, CA), DNA-coated gold particles (bullets) are accelerated to high speed by pressurized helium and are able to overcome physical barriers such as the stratum corneum in the epidermis *(35)* or the actin-rich cuticular plate of inner ear hair cells. This method is suitable for the delivery of mRNA, siRNA, or cDNA to terminally differentiated cells that are difficult to transfect such as neurons, inner ear sensory cells, or cells from internal cellular layers *(33, 36, 37)*. Gene Gun-mediated transfection works well even with postnatal inner ear sensory epithelial explants, can be used to co-transfect two or more different plasmids on the same bullet, and is suitable for delivery of large cDNAs.

Using the Helios® Gene Gun, we successfully transfected hair cells with cDNA expression constructs of GFP-tagged full-length myosin Ic, myosin VI, myosin VIIa, myosin XVa, whirlin, espin, and γ- and β-actin (*4, 6, 7*, and unpublished data). Some of our results with cDNA expression constructs of GFP-tagged myosin XVa, whirlin, and β-actin will be used in this chapter to illustrate the Gene Gun transfection method. Our data show that Helios® Gene Gun–mediated transfection in combination with fluorescence immunostaining and genetic and phenotype analyses of mouse models of human deafness is a valuable tool to elucidate functions of these genes and their encoded proteins.

Inner ear sensory epithelia are populated by many cell types with apical surfaces that have different physical properties. Directly underneath the apical plasma membrane of the sensory hair cells of the organ of Corti is a dense actin meshwork referred to as the cuticular plate. The cuticular plate helps hair cells withstand disturbances due to acoustic stimulations. It also provides support for the rows of stereocilia, which are rigid microvilli-like projections on the apical surface of a hair cell.

Stereocilia are susceptible to damage due to the applied pulse of helium pressure as well as to gold particle bombardment. Meanwhile, the dense cuticular plate is an obstacle to the introduction of gold particles into sensory hair cells. These factors require careful consideration of the many parameters and settings needed for using the Gene Gun to transfect cDNA into sensory hair cells. Variables include the distance between the cartridge with bullets and the targeted tissue, the angle at which bullets strike the cells, the helium pressure applied to propel the bullets toward the tissue, the thickness of the residual liquid layer that covers the tissue during bombardment, the density of bombarding gold particles over the surface area of targeted cells, the purity and concentration of DNA, and the general quality of the cartridges and bullets, which is discussed in Section 3 of this chapter (*see* **Note 1**). This chapter describes in detail the experimental protocol, including preparation of the organotypic cultures of the sensory epithelia of the inner ear from postnatal mice and rats, coating microcarriers with plasmid DNA, cartridge preparation, and bombarding tissues with DNA-coated gold particles accelerated by a pulse of helium gas pressure (*see* **Note 2**).

2. Materials

2.1. Preparation of Inner Ear Sensory Epithelial Explants

1. Experimental animals. Mouse or rat pups of postnatal days 0–4 (*see* **Note 3**).
2. Dissection tools and microscope (*see* **Note 4**).
3. Sterile 60 × 15 mm polystyrene tissue culture dishes (Becton Dickinson and Co., Franklin Lakes, NJ).
4. Leibowitz's L-15 medium without phenol red (Invitrogen, Carlsbad, CA). Store at 4 °C.
5. Sterile MatTek glass bottom Petri dishes (cat. no. P-50G-0-14-F, MatTek Corp, Ashland, MA) (*see* **Note 5** and **Fig. 7.1**).
6. 2.18 mg/mL Cell-Tak cell and tissue adhesive (BD Biosciences, San Jose, CA). Store at 4 °C.
7. Tissue culture grade water (Invitrogen).
8. Dulbecco' Modified Eagle's Medium (DMEM) with high glucose content (4.5 g/L) and 25 m*M* HEPES buffer

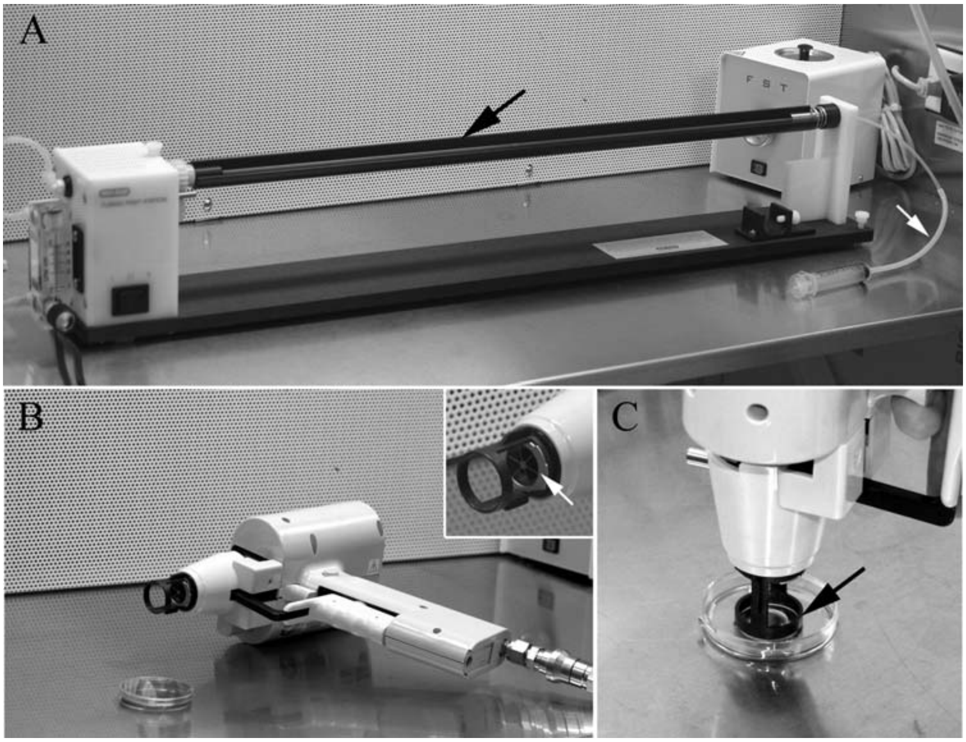

Fig. 7.1. **Bio-Rad Helios® Gene Gun and Tubing Prep Station**. (**A**) Tubing Prep Station with Tefzel tubing inserted into the tubing support cylinder (*black arrow*). The right end (~15 cm) of the Tefzel tubing is sticking out and is connected to the 10 cc syringe with adaptor tubing (white arrow). (**B**) An assembled Gene Gun with a diffusion screen inserted into the barrel. The insert shows a close view of a barrel with a diffusion screen (*white arrow*). Next to the Gene Gun, there is a MatTek glass bottom Petri dish, containing the attached sensory epithelium explant in DMEM. (**C**) Correct placement of the Gene Gun while transfecting inner ear sensory epithelium cultured in a MatTek Petri dish. The plastic ring at the end of the barrel (*black arrow*) is positioned so that the explant appears in the center of the ring. DMEM was removed in preparation for firing.

(Invitrogen) supplemented with 7% (v/v) fetal bovine serum (FBS, Invitrogen). Store at 4 °C (*see* **Note 6**).

9. Sterile microdissecting curette, 12.7 cm, size 3, 2.5 mm (Biomedical Research Instruments, Rockville, MD) (*see* **Note 7**).
10. Tissue culture incubator set at 37 °C and 5% CO_2 (*see* **Note 8**).

2.2. Preparation of Bullets with DNA-Covered Gold Microcarriers

1. 50 μg of plasmid DNA at 1 mg/mL (*see* **Note 9**). Store at −20 °C.
2. Fresh (unopened) bottle of 100% ethyl alcohol. Store in a cabinet for flammable alcohol reagents at room temperature (*see* **Note 10**).
3. 1 *M* $CaCl_2$: Dilute in the DNase, Rnase-free molecular biology grade water from 2*M* $CaCl_2$ molecular biology grade stock solution. Prepared stock solutions can be purchased

from several vendors (e.g., Quality Biological, Inc., Gaithersburg, MD).

4. 1 μm gold microcarriers or tungsten microcarriers (Bio-Rad) (*see* **Note 11** , *(38)*).
5. 20 mg/mL polyvinylpyrrolidone (PVP, Bio-Rad): Weigh out 20 mg of crystallized PVP, add 1 mL of 100% ethanol, and vortex. PVP becomes fully dissolved within 5–10 min at room temperature. Store at 4 °C and use within 1 month (*see* **Note 12**).
6. 0.05 *M* spermidine (cat. no. S0266, Sigma-Aldrich Inc. St. Louis, MO) stock solution: Dilute the content of one ampule (1 g) of spermidine in 13.6 mL of DNase, RNase-free molecular biology grade water to get a 0.5 *M* stock solution. Store this solution as single-use aliquots at −20 °C for 1 month. For a working solution to use in bullet preparation, thaw one aliquot of stock solution, take 5 μL, and add 45 μL of DNase, RNase-free molecular biology grade water to obtain a final concentration of 0.05 *M*. Use the same day (*see* **Note 13**).
7. Two sterile 15 mL conical tubes and sterile 1.5 mL centrifuge tubes.
8. Ultrasonic cleaner (waterbath sonicator) (e.g., Model 50D, VWR International, West Chester, PA) (*see* **Note 14**).
9. Tubing Prep Station (**Fig. 7.1A**) (cat. no. 1652418, Bio-Rad). Clean by wiping with 70% (v/v) ethanol before each use.
10. Nitrogen gas tank, grade 4.8 or higher, and nitrogen regulator (cat. no. 1652425, Bio-Rad). Also, see the Bio-Rad Helios® Gene Gun System instruction manual for nitrogen gas requirements.
11. Tefzel tubing (cat. no. 165-2441, Bio-Rad).
12. Tubing cutter and disposable blades (cat. no. 165-2422 and 165-2423, Bio-Rad).
13. 10 cc syringe with ~12–15 cm of syringe adaptor tubing (**Fig. 7.1A**, white arrow) (Bio-Rad).
14. 20 mL disposable scintillation vials with caps (cat. no. 7451020 Kimble Glass Inc., Vineland, NJ) and desiccating capsules of drycap dehydrators type 11 (Ted Pella, Inc., Redding, CA).

2.3. Helios® Gene Gun Transfection Procedure

1. Helium gas tank grade 4.5 (99.995%) or higher should be used together with a helium pressure regulator (cat. no. 165-2413, Bio-Rad).
2. Helios Gene Gun System, 100/120 V (**Fig. 7.1B**) (cat no. 1652431, Bio-Rad).
3. A diffusion screen (**Fig. 7.1B**, white arrow in the insert) (cat no. 165-2475, Bio-Rad) can be reused with the same DNA preparation (*see* **Note 15**).

4. Inner ear sensory epithelial explants attached to the bottom of a glass bottom MatTek Petri dish (prepared as described in "Methods" section).

2.4. Counterstaining, Immunostaining, and Imaging of Transfected Samples

1. 1X phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}: $1.06\,\text{m}M$ KH_2PO_4, $155.17\,\text{m}M$ NaCl, $2.97\,\text{m}M$ Na_2HPO_4 (*see* **Note 16**). Store at 4 °C.
2. 4% (v/v) paraformaldehyde fixative: Dilute 16% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) 1:4 with 1X PBS (1X PBS is without Ca^{2+} and Mg^{2+} if not specifically mentioned otherwise). Store at 4 °C.
3. 0.5% (v/v) Triton X-100: Dilute 0.25 mL of 100% Triton X-100 (ACROS Organics, New Jersey, USA) in 50 mL of 1X PBS (*see* **Note 17**).
4. Blocking solution: Dilute 0.2 g of bovine serum albumin fraction V, protease-free (Roche Diagnostics, Indianapolis, IN) and 0.5 mL goat serum (Invitrogen) in 10 mL of 1X PBS. Keep refrigerated and use within 48 h. Every time before use, filter the desired volume of blocking solution using a syringe-driven MF membrane filter unit (25 mm in diameter and 22 μm pore size; Millipore Corporation, Bedford, MA) for sterilization of aqueous solutions.
5. Primary antibody to recognize endogenous native or fluorescently tagged newly synthesized protein. For example, to recognize endogenous whirlin or GFP-tagged whirlin, we use polyclonal rabbit anti-whirlin antibody, diluted 1:400 in blocking solution (**Fig. 7.2**, *(6)*). Store at −80 °C.
6. Secondary antibody conjugated to a fluorophore. For example, to bind to polyclonal rabbit anti-whirlin primary antibody (**step 5 of Section 2.4**), we use Alexa 643-conjugated goat anti-rabbit secondary antibody (Invitrogen). Store at 4 °C. Dilute 1:500 in blocking solution at time of use.
7. Rhodamine-phalloidin (Invitrogen). Dilute 1:100 in 1X PBS or blocking solution before use (*see* **Note 18**).
8. A short (146 mm) glass Pasteur pipette (Ted Pella, Inc., Redding, CA) to transfer inner ear sensory epithelial explant from MatTek petri dish to a glass slide to mount. To make a tip opening of the Pasteur pipette wider to accommodate the explant, cut the narrow part of the pipette with glass cutter tool.
9. Anti-fade kit (Invitrogen). Store at −20 °C. Use according to manufacturer's instructions (*see* **Note 19**).
10. LSM510 confocal microscope (Carl Zeiss Inc., Göttingen, Germany) equipped with a 100X, 1.4 numerical aperture objective or another confocal microscope suitable for fluorescence imaging.

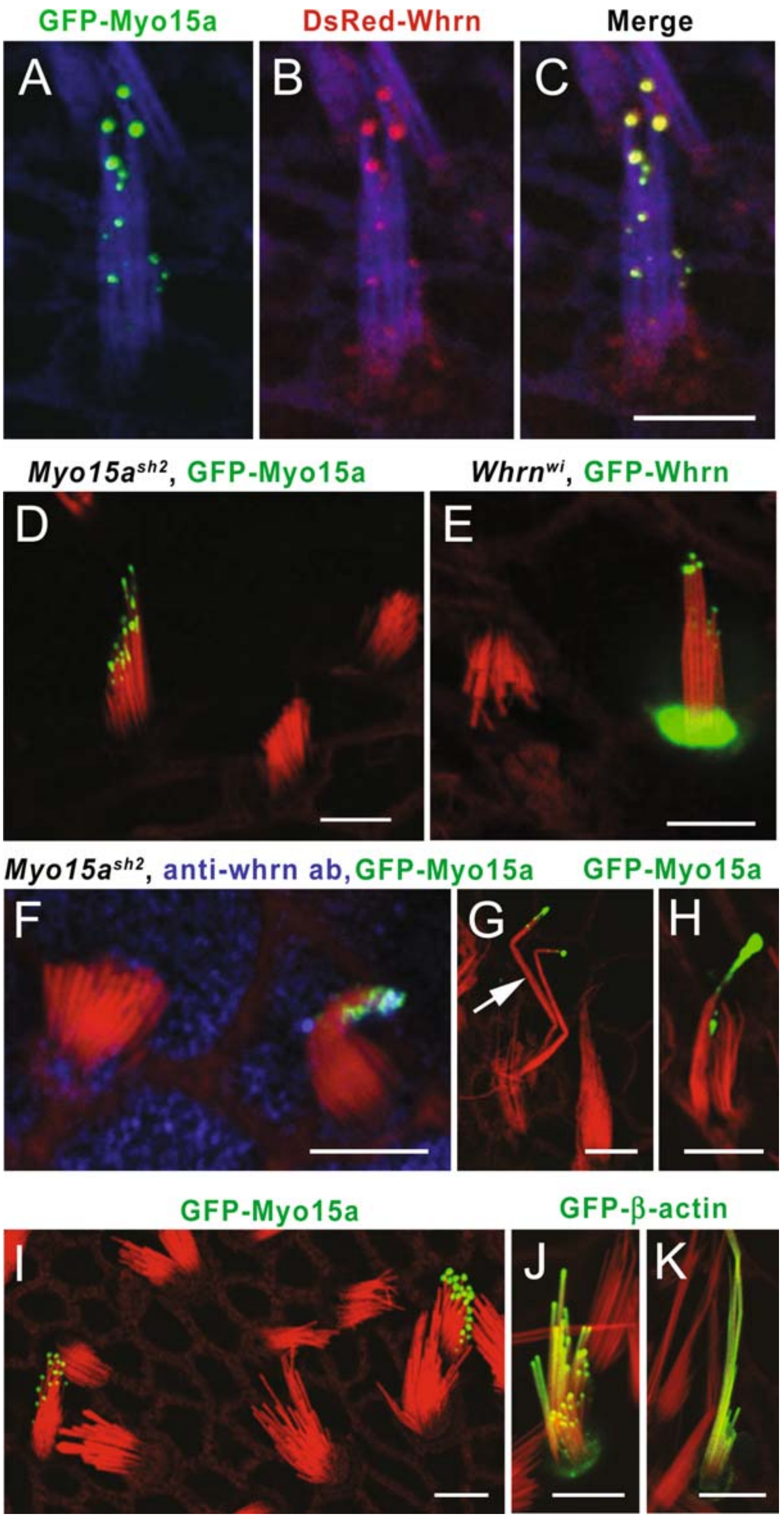

Fig. 7.2. **Gene Gun-transfected vestibular hair cells from organotypic cultures of wild type and mutant mouse inner ear sensory epithelia**. (**A–C**) Simultaneous transfections of GFP-Myo15a and DsRed-Whrn into the vestibular

3. Methods

3.1. Preparation of Inner Ear Sensory Epithelial Explants

1. Prepare MatTek Petri dishes by coating the entire glass bottom of the dish with Cell-Tak diluted 1:6 (v/v) in tissue culture grade water. Let Cell-Tak dry. Immediately before transferring the tissue into the Cell-Tak-covered MatTek Petri dish, wash the dish once briefly with DMEM without FBS (*see* **Note 20**).
2. Dissect sensory epithelia from postnatal day 0–4 (P0–P4) mouse or rat inner ear in a 60-mm sterile cell culture dish with the tissue submerged in L-15 medium. In the case of the organ of Corti, remove spiral ligament and the stria vascularis, remove the tectorial membrane, dissociate the organ of Corti from the modiolus, and then cut the entire organ of Corti into the desired number of pieces. Microdissection of the vestibular sensory epithelia should include removal of utricular and saccular otoconia using a 26-gauge needle.
3. Transfer one or two pieces of the epithelia into the MatTek Petri dish with 2 mL of DMEM supplemented with 7% (v/v) FBS using a microdissecting curette (*see* **Note 7**). Submerge all pieces and gently push them against the surface of the dish coated with Cell-Tak to attach them to the substrate (*see* **Note 21**). Immediately place the dish with attached organotypic culture in an incubator at 37 °C and 5% CO_2. Let the tissue adhere to the dish undisturbed overnight.

Fig. 7.2. (continued) hair cell of a wild type mouse. GFP-myosin XVa (*left*) and DsRed-whirlin (*middle*) accumulate at the tips of stereocilia in direct proportion to each other and to the length of stereocilia. The merged image (*right*) shows overlapping localization of GFP-myosin XVa and DsRed-whirlin. Cytoskeletal actin is visualized using phalloidin 633 (*blue*). (**D**) Restoration of the staircase shape of a stereocilia bundle of a homozygous $Myo15a^{sh2}$ vestibular hair cell 67 h after GFP-Myo15a transfection. Note the short length of stereocilia of non-transfected neighboring $Myo15a^{sh2}$ hair cells. (**E**) Restoration of the staircase shape of the stereocilia bundle in a homozygous $Whrn^{wi}$ vestibular hair cell 48 h after transfection with GFP-Whrn. (**F**) Exogenous GFP-myosin-XVa (*green*) recruits endogenous whirlin stained with anti-whirlin HL5136 antibody (*blue*) to stereocilia tips of a $Myo15a^{sh2}$ in transfected vestibular hair cell. Note that there is no anti-whirlin immunoreactivity in the stereocilia of neighboring non-transfected hair cells (*left*). Images in **A-F** are reproduced from Belyantseva et al., 2005 *(6)*. (**G**) Rat vestibular hair cell stereocilia bundle (transfected with GFP-Myo15a) degenerates as a result of excessive helium pressure and particle bombardment. Two giant, over-elongated, and deformed stereocilia (*arrow*) were observed 40 h post-transfection. The stereocilia bundle of non-transfected neighboring hair cell remains intact. (**H**) Degeneration of a stereocilia bundle transfected with GFP-myosin XVa (GFP-Myo15a) in a mouse vestibular hair cell 26 h post-transfection. There is an enormous accumulation of GFP-myosin XVa at the tips of fused stereocilia. (**I**) Accumulation of variable amounts of GFP-myosin XVa at the tips of stereocilia of two transfected mouse vestibular hair cells from the same sensory epithelium explant 45 h post-transfection. Images is reproduced from Belyantseva et al., 2003 *(4)*. (**J, K**) Different patterns of GFP-β-actin distribution in stereocilia of simultaneously transfected mouse vestibular hair cells, from the same explant 48 h post-transfection. (**J**) GFP-β-actin is mostly at the tips of stereocilia. (**K**) GFP-β-actin is distributed along the length of stereocilia. Cytoskeletal actin is visualized by rhodamine-phalloidin (*red*) in panels **D–K**. Sensory explants were harvested at P2–P5 and transfected the next day. Scale bars: 5 μm.

3.2. Preparation of Bullets with DNA-Covered Gold Microcarriers

1. Weigh out 25 mg of gold microcarrier into a 1.5 mL centrifuge tube.
2. Add 100 μL of 0.05 *M* spermidine (*see* **Notes 9, 13**). Vortex for 10–15 sec, and then sonicate the tube for 30 sec by dipping the tube half-way into the water bath of the sonicator.
3. Add 50 μg of plasmid DNA (50 μL of 1 mg/mL). Vortex briefly (~ 5 sec) to ensure even distribution of DNA in gold suspension (*see* **Note 9**).
4. Add 100 μL of 1 *M* $CaCl_2$ one drop at a time to the tube with DNA. Vortex briefly (~ 3 sec) after each drop. If more than 100 μL of DNA is used, match this volume with the same amount of $CaCl_2$. Incubate the tube at room temperature for 10 min.
5. Cut ~76 cm of Tefzel tubing using scissors. Trim both ends using the Bio-Rad Tubing cutter. Position the Tubing Prep Station so that the nitrogen gas meter and the “ON-OFF” switch for rotation are facing toward you (**Fig. 7.1A**). Insert the tubing into the Tubing Prep Station, leaving about 10 cm of the tube sticking out on the right hand side (**Fig. 7.1A**).
6. Turn “on” the nitrogen gas to 0.3–0.4 L/min and flush the tubing for 10–15 min.
7. Dilute PVP to 50 μg/mL using 100% ethanol. (Add 10 μL of 20 mg/mL of PVP to 4 mL of ethanol in 15 mL conical tube.) (*see* **Note 12**).
8. Microfuge the gold with DNA at 1,000 g for 2 min at room temperature to pellet gold particles. Aspirate excess supernatant using a 1 mL pipette tip, leaving about 20 μL. Resuspend the gold pellet in this residual volume by gently tapping on the lower part of the tube.
9. Wash three times in 1 mL of 100% ethanol. Pellet gold by centrifugation at 1,000–2,000 g at room temperature for 10 sec, aspirate supernatant as in **step 8 of Section 3.2**, resuspend gold, and add 1 mL of 100% ethanol. After the last wash, remove most of the ethanol (*see* **Note 10**).
10. Add 200 μL of the 50 μg/mL PVP in ethanol made in **step 7 of Section 3.2**. Pipette up and down to break up clumps. Transfer the contents of the tube to a 15 mL conical tube. Add another 200 μL of 50 μg/mL PVP in ethanol to the centrifuge tube, repeat the pipetting, and transfer to the same 15 mL tube until all the gold particles are transferred. Bring the final volume to 3 mL with fresh 50 μg/mL PVP in ethanol. Vortex briefly for about 15 sec to ensure an even distribution of gold particles in the suspension. Close the tube and keep inverting it by hand to prevent the gold from clumping.
11. Turn off the nitrogen gas on the Tubing Prep Station. Insert the right end of the Tefzel tubing into the adaptor tubing

(*see* **step 13 of Section 2.2**) attached to an empty 10 cc syringe. Remove the tubing from the apparatus. Remove the cap of the 15 mL tube containing 3 mL of the gold particle suspension (*see* **step 10 of Section 3.2**) and immediately place the left end of the Tefzel tubing at the bottom of this tube. Pull the plunger of the syringe and quickly and consistently draw the gold suspension into the tubing. When the entire volume of gold particle suspension is within the Tefzel tubing, continue drawing the suspension into the tubing to empty ~2–3 cm of the left end segment of the tubing. Make sure that the gold particle suspension is distributed along the Tefzel tubing evenly, without air bubbles, and is not drawn into the adaptor tubing (**Fig. 7.1A**, white arrow). Immediately bring the gold-filled tubing to a horizontal position and slide it, with syringe attached, into the tubing support cylinder (**Fig. 7.1A**, black arrow) of the Tubing Prep Station until the tubing passes through the O-ring.

12. Let the tubing sit undisturbed for 3 min in the tubing apparatus. The gold will settle to the lower side of horizontally positioned tubing.
13. Use the syringe with adaptor tubing (*see* **step 11 of Section 3.2**) attached to the right end of the tube with gold suspension to slowly and consistently pull the supernatant (ethanol) out of the tubing over the course of ~40–45 sec. After all of the ethanol is transferred into the syringe and the connecting tubing, disconnect the syringe with adaptor tubing from the Tefzel tube with gold particles.
14. Turn the rotation switch to the "ON" position on the Tubing Prep Station and rotate the tube with gold particles for 20–30 sec, allowing the gold to uniformly smear on the inside surface of the tube.
15. On the Tubing Prep Station, slowly open the valve on the flowmeter regulating the nitrogen gas to 0.35–0.4 L/min, while rotating the tubing with gold particles for 5 min.
16. Stop rotating the tubing. Turn off the nitrogen gas and remove tubing from the apparatus. Trim the ends of the tubing, which usually are not evenly coated with gold. Cut the tubing into 1.27 cm sections with the Bio-Rad Tubing cutter. These pieces are now the cartridges with bullets.
17. Store these prepared cartridges with bullets in tightly closed scintillation vials (*see* **step 14 of Section 2.2**) with one capsule of drycap dehydrators in each vial (*see* **step 14 of Section 2.2**). The coated gold particles stored at 4 °C are usable for about 1 year (*see* **Note 22**).

3.3. Helios® Gene Gun Transfection Procedure

1. Load bullets with the desired plasmid DNA into the cartridge holder. Leave slot no. 1 empty. Place the cartridge holder into the Gene Gun.

2. Connect the Gene Gun to the helium gas tank via the helium regulator. Open the gas valve and set the pressure to 758.42 kPa (110 psi). Using empty slot no. 1 for firing, discharge the Helios Gene Gun 2–3 times. Be sure that the helium pressure remains stable and does not drop during these trial shots.
3. Switch to slot position no. 2. Insert the diffusion screen into the Helios® Gene Gun barrel as shown in **Fig. 7.1B** (*see* **Note 15**).
4. Remove a dish with the inner ear sensory explants from the incubator and place it in a sterile laminar flow hood.
5. Aspirate culture medium (as much as possible) from the inner ear organotypic culture attached at the bottom of the MatTek Petri dish (**Fig. 7.1B,C**). Immediately position the plastic ring, located at the end of the Helios® Gene Gun barrel (**Fig. 7.1C**, black arrow), at the bottom of the Petri dish so that the targeted tissue is located in the center of the ring, and the Gene Gun barrel is perpendicular to the dish bottom. Discharge the Gene Gun (**Fig. 7.1C**; *see* **Note 23**).
6. Immediately add 2 mL of fresh DMEM medium containing 7% FBS to the dish. Without delay, place the dish in the incubator for the desired number of hours/days.
7. Repeat steps 1 through 5 for the other dishes with organotypic culture using fresh bullet cartridges in the consecutive slots of the same cartridge holder.

3.4. Immunostaining and Imaging of Transfected Samples

1. Wash cultures two times in cold 1X PBS (*see* **Note 16**).
2. Fix in 4% paraformaldehyde for 30 min to 1 h at room temperature or overnight at 4 °C.
3. Wash four times with 1X PBS for 5 min.
4. Permeabilize in 0.5% Triton X-100 for 10–15 min (*see* **Note 17**).
5. Wash four times with 1X PBS for 5 min.
6. Incubate in blocking solution (2% BSA and 5% goat serum in 1X PBS) for 30 min.
7. Incubate in primary antibody diluted in blocking solution for 1–2 h at room temperature or overnight at 4 °C (*see* **Note 18**).
8. Wash four times with 1X PBS for 5 min.
9. Incubate simultaneously for 20 min at room temperature in secondary antibody diluted in blocking solution (*see* **step 6 of Section 2.4**) and phalloidin conjugated to a particular fluorophore (Invitrogen) and diluted 1:100 in blocking solution (*see* **step 7 of Section 2.4**) (*see* **Note 18**).
10. Wash four times in 1X PBS.
11. Using a 26-gauge needle, lift the inner ear explant off the glass bottom of the MatTek Petri dish. Transfer the explant to a glass slide by carefully drawing it into a short (146 mm)

glass Pasteur pipette (*see* **Section 2.4, step 8**). Position the sensory epithelium on the glass slide with stereocilia facing up; remove the surrounding liquid as much as possible before adding anti-fade mounting medium. Immediately apply a drop of mounting medium. Mount the tissue using the ProLong Antifade kit (Invitrogen) according to the manufacturer's instructions (*see* **Note 24**).

12. Keep slides protected from light in a slide box overnight to let the mounting media solidify (*see* **Note 25**). Acquire images on the next day using a confocal microscope equipped with a 100X, 1.4 numerical aperture objective.

3.5. Biolistic Transfection of Inner Ear Sensory Epithelium: Interpretation of Results

The advantages and disadvantages of the described method can be illustrated by analyzing the data obtained from Gene Gun–mediated transfections of GFP-myosin XVa and/or DsRed- or GFP-whirlin into hair cells of inner ear sensory epithelial explants obtained from wild-type and mutant mice (*6*). In wild-type hair cells, the unconventional motor protein myosin XVa and the PDZ domain-containing protein whirlin localize together at the tips of stereocilia *(4, 6, 7, 39)*. We used a Helios® Gene Gun to co-transfect two expression vectors. Gold particles were coated with two different cDNAs at a 1:1 ratio of molecules. GFP-myosin XVa and DsRed-whirlin on the same gold bullet were co-transfected into wild-type hair cells. Both corresponding epitope-tagged proteins were localized to the tips of stereocilia *(6, 7)*. These data complemented observations of the endogenous myosin XVa and whirlin at the tips of stereocilia as revealed by immunofluorescence and, most importantly, demonstrated that GFP and DsRed epitope tags do not interfere with proper targeting or function of these two proteins (**Fig. 7.2A–C**). Moreover, over-expression of GFP-myosin XVa in stereocilia of wild-type hair cells causes distention of stereocilia tips due to an accumulation of an excessive amount of GFP-myosin XVa. No over-elongation of stereocilia of wild-type hair cells due to over-expression of myosin XVa was observed *(4, 7)*.

Myosin XVa mutant (*Myo15a*^{sh2}) and whirlin mutant (*Whrn*^{wi}) strains of deaf mice have hair cells with abnormally short stereocilia bundles that fail to elongate to a normal length due to mutations of these genes *(4, 6, 40–42)*. Gene Gun–mediated transfections of wild-type GFP-myosin XVa and wild-type GFP-whirlin into the hair cells of sensory epithelial explants from the corresponding mutant mice resulted in the restoration of a normal length of stereocilia bundles (**Fig. 7.2D–E**). Moreover, using Gene Gun–mediated transfections of domain-deletion constructs of myosin XVa and whirlin cDNAs into *Myo15a*^{sh2} and *Whrn*^{wi} hair cells, we found that these two proteins interact *in vitro* through the C-terminal PDZ ligand of myosin XVa and the third PDZ domain of whirlin. This interaction allows myosin XVa to deliver whirlin to the tips of stereocilia. Using an anti-whirlin specific antibody in combination with Gene Gun transfection, we found that exogenous wild-type GFP-myosin XVa "reawakens" the elongation process in the abnormally short *Myo15a*^{sh2} hair cell stereocilia by recruiting endogenous whirlin to stereocilia tips **(Fig. 7.2F)**.

3.5.1. Advantages

In lipofection, viral mediated transfection, electroporation, and calcium phosphate precipitation a transfected cell may receive a very small amount of fluorescently tagged cDNA expression construct. In this case, the amount of synthesized fluorescently tagged protein might be below the threshold of detection by fluorescence microscopy, and this cell might be indistinguishable from and mistaken for untransfected control cells with background fluorescence. Nevertheless, a low level of the epitope-tagged protein may subtly alter the phenotype of the cell. One of the advantages of Gene Gun transfection is that you can usually see a gold particle inside the body of a transfected cell while imaging, thereby distinguishing a transfected cell from adjacent cells that lack such a particle and may serve as an untransfected control. Thus, true control cells can be identified and distinguished from transfected cells. Moreover, in our experiments (*see* **Section 3.5**), stereocilia undergo elongation only when wild-type GFP-tagged myosin XVa or whirlin is transfected into hair cells of *Myo15a*^{sh2} and *Whrn*^{wi}, respectively. In contrast, stereocilia bundles of non-transfected hair cells from the same explant remain short, because they are deficient for functional myosin XVa (**Fig. 7.2D,F**) or whirlin (**Fig. 7.2E**). Thus, Gene Gun transfections provide the opportunity to quantitatively measure the induced elongation of stereocilia, by comparing the lengths of restored stereocilia of transfected hair cells to the lengths of short hair bundles of non-transfected neighboring control cells. These types of experiments, with the Helios® Gene Gun, can reveal the importance of specific proteins to key developmental events, as demonstrated by the importance of myosin XVa and whirlin to the differential elongation of stereocilia during hair bundle morphogenesis *(6)*.

3.5.2. Disadvantages

Helios® Gene Gun–mediated transfections of cultured inner ear sensory epithelia have limitations. First, hair cells survive at most for 2 weeks in culture once a mouse organ of Corti is explanted at postnatal day 0 through day 4 (P0–P4). Second, stereocilia are sensitive to mechanical disturbances and are easily damaged by a pulse of helium gas pressure, triggering hair cell degeneration. An early sign of degeneration is an abnormal hair bundle shape (**Fig. 7.2G–H**). Stereocilia show unrestrained elongation and/or fusion and some stereocilia proteins localize abnormally, such as GFP-tagged proteins introduced via cDNA constructs (**Fig. 7.2G–H**). Thus, care should be exercised in interpreting the results if the hair bundle of a transfected cell looks abnormal, especially in explants from mutant mice. For example, an over-elongated or disorganized stereocilia bundle of a transfected *Myo15a*^{sh2} hair cell may likely result from degeneration rather than over-expression of GFP-myosin XVa. Moreover, individual stereocilia may elongate abnormally within a short stereocilia bundle, even in non-transfected *Myo15a*^{sh2} hair cells that lack functional myosin XVa (*see* cover image of *6, 7*). Therefore, the presence of abnormal hair bundles with over-elongated stereocilia should not be interpreted as a result of myosin XVa-GFP over-expression in transfected wild-type hair cells (e.g., **Fig. 7.2G–H**). Rather, in both transfected and non-transfected *Myo15a*^{sh2} hair cells, over-elongation of stereocilia can be interpreted as a consequence of degeneration caused by mechanical damage. In general, hair cell degeneration may be a secondary effect of a variety of environmental or genetic insults to the sensory epithelium, including mechanical damage due to helium gas pressure or noise, gold particle bombardment, culture conditions for a prolonged period of time, and/or a gene mutation.

Third, dissimilar amounts of plasmid DNA on the gold microcarriers may affect data interpretation. While only one gold particle penetrates a hair cell for most transfections, transfected hair cells may show different levels of GFP-tagged protein expression at a specific point in time. **Fig. 7.2I** shows different levels of myosin GFP-XVa accumulation at the stereocilia tips of hair cells from the same explant 45 h post-transfection. A second example is GFP-β-actin, which first appears at the tips of stereocilia, then supposedly incorporates into actin filaments and treadmills toward the apical surface of the hair cell at a particular rate *(3, 5)*. Hair cells from the same explant simultaneously transfected with full-length GFP-β-actin may show dissimilar amounts and different distribution patterns of this protein within stereocilia. These differences in GFP-β-actin distribution in stereocilia, after Gene Gun–mediated transfection, are similar to differences described for viral infection/delivery of GFP-β-actin to hair cells *(11)*. **Figure 7.2J–K** shows Gene Gun–transfected hair cells from the same explant. Approximately 72 h post-transfection, GFP-β-actin is present primarily at the tips of the stereocilia in one hair cell (**Fig. 7.2J**), while GFP-β-actin highlights most of the stereocilia length of another hair cell (**Fig. 7.2K**). The time of appearance and the amount of GFP-tagged protein visualized in a transfected cell are probably related to the amount of DNA transfected into a cell. Therefore, it is important to repeat the above-described experiments (independent determinations each with replicas) to document the range of variation in the kinetics of appearance and localization of the tagged protein. In the absence of such data, one should view any interpretations of time-sensitive expression of GFP-tagged proteins with some skepticism. Rigorous evaluation and accurate interpretation of the data from Gene Gun–mediated transfections may require, for example, immunofluorescence to confirm correct localization of GFP-tagged proteins and genetic analyses of the phenotype of relevant mutant mice.

4. Notes

1. Other Gene Gun models, including Accell®, have been developed by Aurogen, Inc., a Bio-Rad collaborator (*see* also Helios® Gene Gun System Instruction Manual from Bio-Rad, which is available online at http://www.bio-rad.com/LifeScience/pdf/Bulletin_9541.pdf). Cell penetration, gene expression, and other parameters vary with the model of the Gene Gun. Therefore, users must be careful to optimize the operating parameters for their particular model. O'Brien and Lummis *(37)* developed a modified barrel for the Bio-Rad handheld Helios® Gene Gun, which reportedly improves the penetration of gold particles into cultured brain slices and allows the use of lower gas pressures without the loss of transfection efficiency. This modified Gene Gun barrel is available from Modolistics (see http://www2.mrc-lmb.cam.ac.uk/personal/job/index.html). However, there are no reported data on the use of this modification in transfections of inner ear sensory epithelial explants. Meanwhile, we have optimized transfection conditions, taking into account all of the above-mentioned variables for our application, using the original Bio-Rad Helios® handheld Gene Gun.
2. You can find information about the assembly, operation, maintenance, spare parts, and general optimization of particle delivery in the Helios® Gene Gun System Instruction Manual from Bio-Rad. Also, there is a helpful troubleshooting section.
3. All experimental animals should be handled according to the protocols of the Instituional Animal Care and Use Committee.
4. All microdissections of the inner ear sensory epithelia should be carried out under sterile conditions using autoclaved instruments. Preferably, a clean dissection microscope should be placed in a laminar flow hood.
5. Glass-bottomed Petri dishes of different diameters can also be used and purchased from other companies (e.g., World Precision Instruments, Inc., Sarasota, FL or Electron Microscopy Sciences).
6. DMEM/F12 media supplemented with 7% (v/v) FBS can also be used.

7. This microdissecting curette will allow you to transfer pieces of sensory epithelia submerged in a limited volume of L-15 media. Pieces of the sensory epithelia can also be transferred using a glass Pasteur pipette (*see* **step 8 of Section 2.4**) attached to a pipette holder (e.g., cat. no. 378980000, A. Daigger & Co., Vernon Hills, IL), which allows you to control the release of liquid from the pipette tip. To transfer your specimen using this pipette, aspirate in the specimen with some L-15 media, let the specimen settle down toward the opening of the pipette (you can help this along by tapping gently on the glass pipette), and then touch the surface of the liquid (DMEM), where you want to transfer your specimen, with the tip of the glass pipette. The specimen will be released into the dish containing DMEM with minimum contamination by L-15 media.
8. It is important to frequently check the water level in the incubator tray to maintain an appropriate humidity level.
9. pAcGFP1-Actin vector is available from BD Biosciences (cat. no. 632453). To use a different vector with your cDNA of interest, you can purchase a Quantum Prep Plasmid Miniprep Kit (100 preps, Bio-Rad, cat. no. 7326100) or Qiagen's QIAfilter plasmid midi kits (Qiagen, Valencia, CA, cat. no. 12243, *see* also *4, 6*) to prepare plasmid DNA of a high purity suitable for Helios® Gene Gun transfection. To use more than 100 μL of a less concentrated plasmid DNA preparation, match the volume of spermidine, but try to avoid volumes larger than 150 μL. More concentrated plasmid DNA (greater then 1.0 mg/mL) may cause gold particles to cluster. It is also crucial to use purified plasmid DNA since impure plasmid DNA may result in poor transfection and/or gold particle clustering. After purification, DNA should be diluted to 1 mg/mL in molecular biology grade water (*see* also *31, 37*).
10. It is very important that the ethanol is free of water (200 proof). A fresh bottle of 100% ethanol should be opened on the day of use in bullet preparation procedures.
11. The size of the microcarrier should be optimized for the particular application, cell types, etc. It is possible to use tungsten particles instead of gold. They are less expensive, but be aware that tungsten can oxidize and may be toxic to the cells *(38)*.
12. Old PVP may cause uneven gold coating of the Tefzel tubing, inefficient release of gold particles during the shot, lower tissue penetration, and reduced transfection efficiency. Do not keep diluted PVP for more than 1 month. The concentration of PVP should be optimized for each particular instrument and application. Crystallized PVP is hygroscopic. Store it at room temperature in a tightly closed desiccated vial.

13. Spermidine solution should be sterile filtered using an 0. 22 μm pore filter, if sterile solution is necessary. Spermidine deaminates with time; solutions should be stored frozen. Old spermidine may cause poor precipitation of DNA onto the gold particles and subsequently reduce the transfection efficiency. Do not keep spermidine for more than 1 month even at −20 °C.
14. O'Brien and Lummis recommend omitting the sonication step *(31, 37)*. However, sonication seems to be efficient in mixing spermidine with gold particles and keeping gold particles in suspension. In the protocol described in this chapter, sonication is omitted only in steps when DNA is added to gold particles to avoid the possible destructive effect of sonication on DNA.
15. The use of a Bio-Rad diffusion screen reduces the damage to the inner ear sensory epithelia and especially to the hair cell stereocilia bundles by reducing the density of the gold particles in the center of the shot during bombardment. After repeatedly firing the Gene Gun, gold particles build up on the center of the diffusion screen, which may become gold colored. Use a dedicated diffusion screen for each plasmid DNA used for firing to avoid cross-contamination. A dedicated diffusion screen exclusively used for a particular plasmid DNA can be reused many times and does not require frequent autoclaving. However, it is necessary to clean diffusion screens before using them to fire bullets coated with a different plasmid DNA. Diffusion screens can be cleaned by soaking in 70% or 100% ethanol and by sterilizing them in an autoclave that uses only distilled water.
16. You can use 1X PBS containing Ca^{2+} and Mg^{2+} if the presence of these ions is required.
17. 100% Triton X-100 is a viscous solution. It is useful to prepare a 1% stock solution by adding 500 μL of 100% Triton X-100 to 49.5 mL of 1X PBS. Before introducing 100% Triton X-100 into a 1 mL pipette tip, cut off about 1 cm of the tip to make a wider opening. This will help to draw a viscous solution in and out of the pipette tip.
18. Rhodamine-phalloidin and other phalloidin conjugates can be used to visualize filamentous actin. Phalloidin 633 can be used to highlight the actin cytoskeleton when cells are co-transfected using two cDNA plasmids tagged with GFP and dsRed. In this case, no primary and secondary antibodies are required.
19. If component B is still too viscous at room temperature, warm it to 37 °C for 30 min before use.
20. A higher concentration of Cell-Tak may improve the adhesion of the sensory epithelia but it can be toxic to hair cells. While diluting Cell-Tak in a 1.5 mL centrifuge tube, make sure you

use it immediately as Cell-Tak quickly adheres to the walls of the tube. Also, you can prepare your dishes using rat tail collagen, type I (Upstate, Lake Placid, NY) *(1, 2)*. Alternatively, you may choose to attach your sample directly to a glass surface not covered with any substrate. In this case, use DMEM without serum during the attachment period.

21. If you have problems with tissue attachment, try DMEM without serum for several hours or overnight. The next morning change the media to DMEM with 7% (v/v) FBS. Also, the freshly dissected tissues seem to adhere to Cell-Tak better than tissues kept in L-15 media for more than ~10 min after microdissection. DMEM/F12 medium (Invitrogen) with 7% (v/v) FBS seems to be better for rat inner ear organotypic culture.
22. Alternatively, to store cartridges you can use tightly closed 15 mL conical tubes, each with one capsule of dehydrator as described in **step 14 of Section 2.2**. Some preparations of plasmid DNA coated onto gold particles were used successfully for transfections after more than two years of storage with proper desiccation.
23. To prevent culture contamination, wipe the plastic ring of the end of a barrel (**Fig. 7.1C**, black arrow) with 70% ethanol after each shot. It is advisable to wear ear protection (earmuffs, cat. no. 56219268, VWR, or earplugs, cat. no. 56610680) when firing the Gene Gun.
24. It is important to remove the 1X PBS from the slide as much as possible without over-drying the sample before adding a drop of Antifade solution. Residual liquid around the sample will interfere with anti-fade properties of the mounting media. In general, samples should not be allowed to dry out at any time during the immunostaining and mounting procedures.
25. You can use clear nail polish to seal the perimeter edge of a coverslip onto a slide. (This step is not necessary if you intend to keep your slide for less than one week). Let it dry before observing the slide under a confocal microscope. For long-term storage up to a few months, store slides at 4 °C with several capsules of desiccant (Ted Pella, Inc.) in the slide box.

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