Chapter 1

Helios® Gene Gun-Mediated Transfection of the Inner Ear Sensory Epithelium: Recent Updates

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Abstract

The transfection of vertebrate inner ear hair cells has proven to be challenging. Therefore, many laboratories attempt to use and improve different transfection methods. Each method has its own advantages and disadvantages. A particular researcher's skills in addition to available equipment and the type of experiment (in vivo or in vitro) likely determine the transfection method of choice. Biolistic delivery of exogenous DNA, mRNA, or siRNA, also known as Helios® Gene Gun-mediated transfection, uses the mechanical energy of compressed helium gas to bombard tissue with micron- or submicron-sized DNA or RNA-coated gold particles, which can penetrate and transfect cells in vitro or in vivo. Helios® Gene Gun-mediated transfection has several advantages: (1) it is simple enough to learn in a short time; (2) it is designed to overcome cell barriers even as tough as plant cell membrane or stratum corneum in the epidermis; (3) it can transfect cells deep inside a tissue such as specific neurons within a brain slice; (4) it can accommodate mRNA, siRNA, or DNA practically of any size to be delivered; and (5) it works well with various cell types including non-dividing, terminally differentiated cells that are difficult to transfect, such as neurons or mammalian inner ear sensory hair cells. The latter advantage is particularly important for inner ear research. The disadvantages of this method are: (1) low efficiency of transfection due to many variables that have to be adjusted and (2) potential mechanical damage of the tissue if the biolistic shot parameters are not optimal. This chapter provides a step-by-step protocol and critical evaluation of the Bio-Rad Helios® Gene Gun transfection method used to deliver green fluorescent protein (GFP)-tagged full-length cDNAs of myosin 15a, whirlin, β-actin, and Clic5 into rodent hair cells of the postnatal inner ear sensory epithelia in culture.

Key words Biolistic transfection, Gene gun, Inner ear, Hair cell, Stereocilia, Myosin, Whirlin, Actin, Clic5, Immunofluorescence, GFP

1 Introduction

During the last few years, there are reports of successful transfections of inner ear hair cells using different methods. One new method, "injectoporation" is just at the beginning of its evaluation by different laboratories [1] and appears to be promising. The technique of intrauterine electroporation is efficient for hair cell transfection, but is not commonly used, likely because of the

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special skills required to perform survival surgery on small rodents [2]. Typically, local injections of non-viral and viral vectors into middle or inner ear is a method of choice for transfecting inner ear cells in vivo, to attempt restoring hearing function in mutant mice by gene therapy [3–7]. Electroporation in vitro, of embryonic and early postnatal organ of Corti explants, is a method of choice in some laboratories [8–14], whereas others have used Helios Gene Gun transfection [15–25]. This chapter is an update on the Gene Gun transfection technique and its place in relation to other methods of transfection of the inner ear sensory epithelia.

Mammalian inner ear hair cells are terminally differentiated, non-dividing cells located within the sensory epithelia of the auditory (organ of Corti) and vestibular periphery (utricular macula, saccular macula, and three cristae ampullares). Hair cells are polarized cells with a cylindrical or pear-like cell body and an apically positioned cuticular plate, which is composed of a dense meshwork of actin. Tight junctions interconnect the apical surfaces of hair cells with surrounding supporting cells. These structural peculiarities of hair cells and their cellular environment may contribute to the ineffectiveness of conventional transfection techniques such as lipofection [26].

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 enter a cell [27, 28]. Electroporation causes a brief increase in membrane permeability after which the membrane quickly reseals. This method is effective with a variety of cell types and species and is used in many applications, including transfection in vivo of embryonic mouse brain [29] and transfection in vitro of immature hair cells from embryonic inner ear explants [13]. One disadvantage of this method is the exposure of the targeted and non-targeted cells to potentially damaging current and electrolysis-generated changes of pH, which may activate stress responses in hair cells. Excessive cell damage and death was a long-standing concern [27] until electroporation devices were improved [28].

A microinjection method of delivering exogenous DNA into a cell, although precise, is labor-intensive. A fully automated robotic system for microinjection was developed and used in zebrafish embryos [30]. Recently, an injectoporation method, combining microinjection of a solution into the sensory epithelium followed quickly by electroporation, was described for the transfection of the mouse organ of Corti [1]. This method of microinjection followed by electroporation was described previously for transfection of planarians, insect larva, and adult insect brain in vivo [31–34]. Xiong and co-authors [1] adopted this method to transfect auditory hair cells in postnatal mouse organ of Corti explants from postnatal day 0 to postnatal day 4. This method appeared to be efficient, although effectiveness is decreased with the age of the postnatal mouse organ of Corti explant [1]. The injectoporation procedure requires microinjection skills under an upright microscope and seems well suited

for electrophysiology laboratories that study functional responses of transfected cells. One drawback of this procedure is the use of antibiotics in the culture media. These drugs are essential to avoid contamination during the prolonged exposure of cultured explants to ambient air, while positioning electrodes and micropipettes within the petri dish filled with culture media and perform microinjection of tissue under the microscope. Another drawback is the potential damage by micropipettes of cell–cell junctions during microinjections, which may affect Ca^{2+} and other signals between hair cells and supporting cells. Avoiding such damage is essential, when studying hair cell innervation or hair cell and Deiters' cell junctions.

The Helios Gene Gun-mediated transfection method of DNA delivery with submicron-sized particles (microcarriers) accelerated to high velocity was developed in the late 1980s by Sanford, Johnston and colleagues [35–38]. This biolistic method was designed to circumvent difficulties in transfecting plant cells with cell walls that prevent simple diffusion and/or internalization of material or vesicles from the cell surface [36]. Subsequently, this method was shown to be applicable to mammalian cells [37]. In the early 1990s, it was used to deliver exogenous DNA to the tissue of a live mouse [38–40]. 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 [35, 37, 40, 41, 43], and in vivo for intradermal vaccination of human and animals using DNA and mRNA vaccines [44, 45]. In the BioRad hand-held Helios® Gene Gun delivery system (BioRad 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 [46] or the actin-rich cuticular plate of inner ear hair cells [15]. 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 [44, 47, 48]. It works well with postnatal inner ear sensory epithelial explants [15–18]. This method can be used to co-transfect two or more different plasmids on the same bullets [18]. It is also suitable for delivery of large cDNAs that do not fit in the limited space of a viral vector, for example. Recently, Helios Gene Gun transfection was combined with live cell imaging, to examine whether or not F-actin core treadmills in hair cell stereocilia [49], as proposed previously [15, 17]. Consistent with the results of a study that shows slow protein turnover in hair cell stereocilia using multi-isotope imaging mass spectrometry [50], the study of gene gun-transfected live hair cells of postnatal mouse utricle reveals stable filamentous actin cores with turnover and elongation restricted to stereociliary tips [49].

Over the last 10 years, we successfully transfected hair cells with cDNA expression constructs of GFP-tagged full-length

myosin Ic, myosin VI, myosin VIIa, myosin 15a, whirlin, espin, γ- and β-actin, and Clic5 using the Helios® Gene Gun [15, 16, 18, 19, 51 and unpublished data]. Some of the data from these papers will be used in this chapter to illustrate the versatility of the Gene Gun transfection method. Our data show that Helios® Gene Gun-mediated transfection is a valuable tool to elucidate the function of "deafness" genes and their encoded proteins, when utilized in combination with fluorescence immunostaining as well as genetic and phenotype analyses of mouse models of human deafness.

Various cell types populating inner ear sensory epithelia have apical surfaces with different physical properties. Directly underneath the apical plasma membrane of sensory hair cells of the organ of Corti is a dense actin meshwork referred to as the cuticular plate. The rootlet of each stereocilium extends into the cuticular plate, which provides a support for the stereociliary bundle [52, 53]. Each auditory stereociliary bundle in mammals is composed of two to three rows of stereocilia, which are mechanosensory microvilli-like projections indispensable for normal hearing function.

Stereocilia may be damaged by the pulse of helium pressure as well as by gold particle bombardment. On the other hand, the dense cuticular plate is an obstacle to the introduction of gold particles into sensory hair cells, which requires a substantial pressure pulse. These factors require careful consideration of the many parameters and settings needed for using the Gene Gun to transfect cDNA into sensory hair cells. The variables to be considered include: (1) the distance between the cartridge with bullets and the targeted tissue, (2) the angle at which bullets strike the cells, (3) the helium pressure applied to propel the bullets toward the tissue, (4) the thickness of the residual liquid layer that covers the tissue during bombardment, (5) the density of bombarding gold particles over the surface area of targeted cells, (6) the purity and concentration of DNA, (7) and the general quality of the cartridges and bullets (*see* Subheading 3 and **Note 1**). The details of the experimental protocol described in this chapter include: (1) preparation of organotypic cultures of the sensory epithelia of the inner ear from postnatal mice and rats, (2) coating microcarriers with plasmid DNA, (3) cartridge preparation, and (4) bombarding tissues with these DNA-coated gold particles accelerated by a pulse pressure of helium gas (*see* **Note 2**).

2 Materials

2.1 Preparation of the 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).

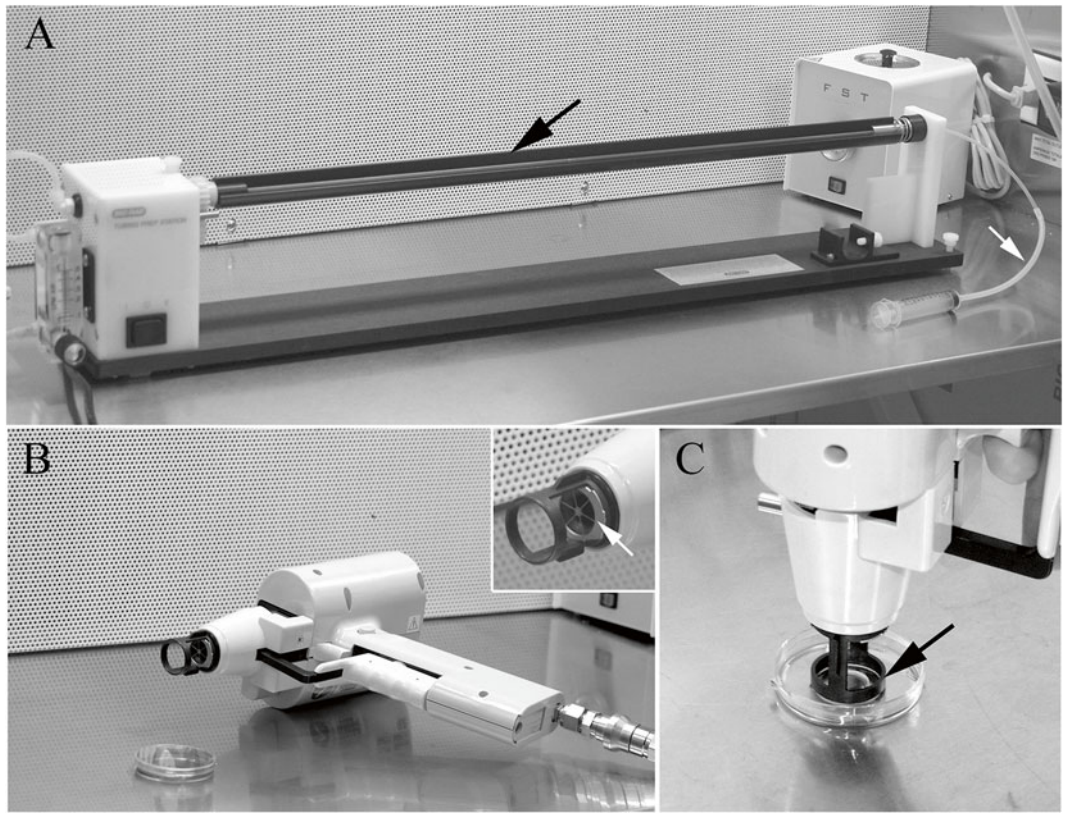

Fig. 1 Bio-Rad Helios® Gene Gun and Tubing Prep Station. (**a**) Tubing Prep Station with Tefzel tubing inserted into the tubing support cylinder (*black arrow*). The right end (~15 cm) of the Tefzel tubing is sticking out and is connected to the 10 cc syringe with adaptor tubing (*white arrow*). (**b**) An assembled Gene Gun with a diffusion screen inserted into the barrel. The insert shows 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

4. Leibowitz's L-15 medium without phenol red (Invitrogen, Carlsbad, CA). Store at 4 °C.
5. Sterile MatTek glass bottom Petri dishes (MatTek Corp, Ashland, MA) (*see* **Note 5** and Fig. 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's Modified Eagle's Medium (DMEM) with high glucose content (4.5 g/L) and 25 mM HEPES buffer (Invitrogen) supplemented with 7 % (v/v) fetal bovine serum. 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 at room temperature in a cabinet for flammable reagents (*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 or 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 (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, Chesten, PA) (*see* **Note 14**).
9. Tubing Prep Station (Fig. 1a) (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 (Bio-Rad). Also, see the Bio-Rad Helios® Gene Gun System instruction manual for nitrogen gas requirements.
11. Tefzel tubing (Bio-Rad).
12. Tubing cutter and disposable blades (Bio-Rad).
13. 10 cc syringe with ~12–15 cm of syringe adaptor tubing (Fig. 1a, white arrow) (Bio-Rad).
14. 20 mL disposable scintillation vials with caps (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 and a helium pressure regulator (Bio-Rad).
2. Helios Gene Gun System, 100/120 V (Fig. 1b) (Bio-Rad).
3. A diffusion screen (Fig. 1b, white arrow in the insert) (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 Subheading 3).

2.4 Counterstaining, Immunostaining, and Imaging of Transfected Samples

1. 1× Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}: 1.06 mM KH_2PO_4, 155.17 mM NaCl, 2.97 mM 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 1× PBS (1× 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 1× 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 1× 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. 2, [18]). Store at –80 °C.
6. Secondary antibody conjugated to a fluorophore. For example, to bind to polyclonal rabbit anti-whirlin primary antibody (Subheading 2.4, **step 5**) we use Alexa 643-conjugated goat anti-rabbit secondary antibody (Invitrogen). Store at 4 °C. Dilute 1:500 in blocking solution at the time of use.
7. Rhodamine-phalloidin (Invitrogen). Dilute 1:100 in 1× 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 glasscutter tool.
9. ProLong Gold Antifade Mountant (Invitrogen). Store and use according to the manufacturer's instructions (*see* **Note 19**).
10. Confocal microscope of any brand suitable for fluorescence imaging. For example, LSM510 or LSM780 confocal microscope (Carl Zeiss Inc., Göttingen, Germany) equipped with a 63× or 100×, 1.4 numerical aperture objective.

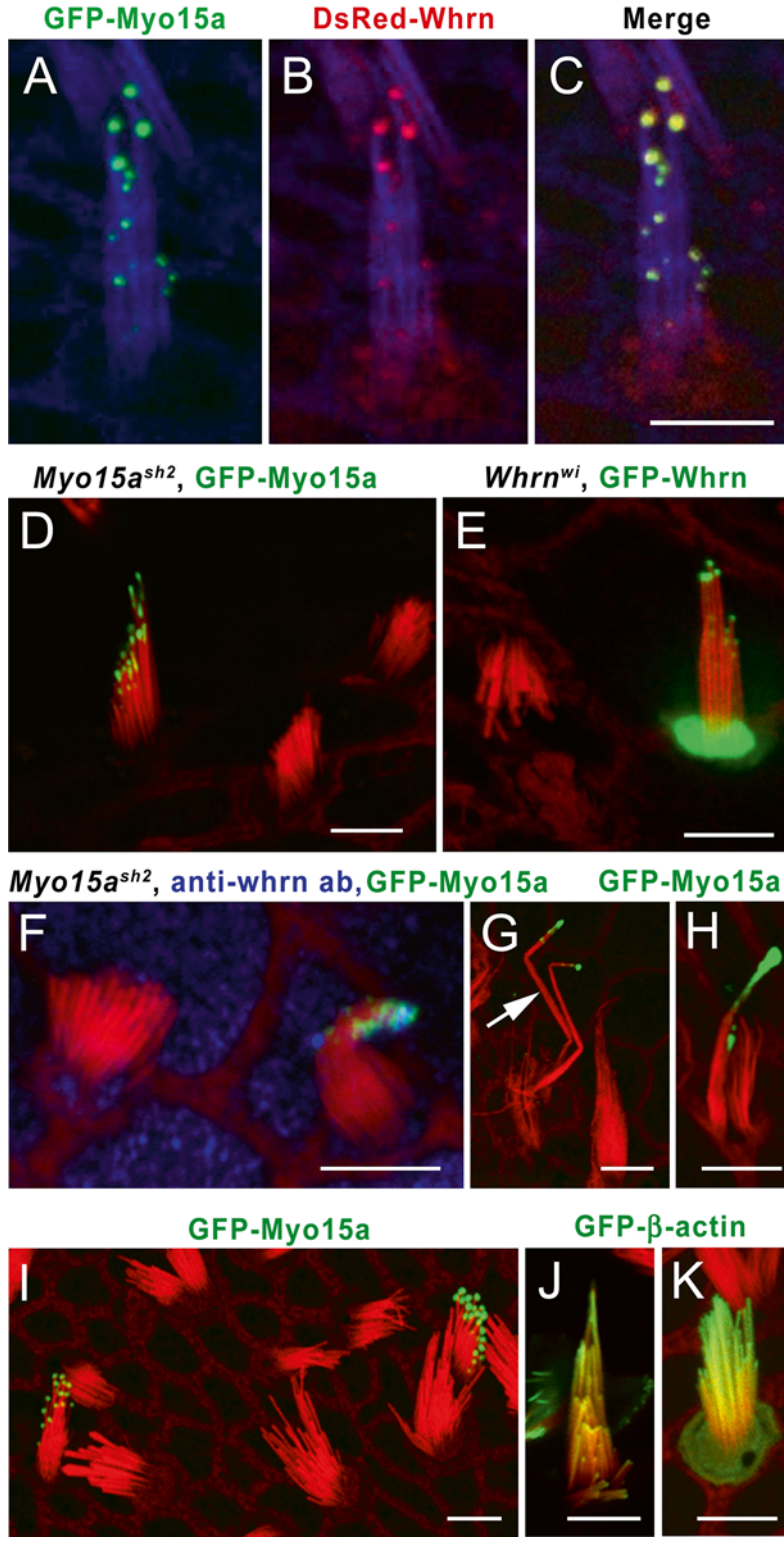

Fig. 2 Gene Gun transfected vestibular hair cells from organotypic cultures of wild-type and mutant mouse inner ear sensory epithelia. (**a**–**c**) Simultaneous transfection of GFP-Myo15a and DsRed-Whrn into the vestibular hair cell of a wild-type mouse. GFP-myosin 15a (*left*) and DsRed-whirlin (*middle*) accumulate at the tips of

3 Methods

3.1 Preparation of the 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, keeping the tissue submerged in L-15 medium. In the case of the organ of Corti, remove the spiral ligament and the stria vascularis, remove the tectorial membrane, using a 26-gauge needle 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 gentle removal of utricular and saccular otoconia, calcium-based crystals sitting on top of the sensory epithelia, 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 while remaining undisturbed overnight.

Fig. 2 (continued) stereocilia in direct proportion to each other and to the length of stereocilia. Merged image (*right*) shows overlapping localizations of GFP-myosin 15a and DsRed-whirlin. Cytoskeletal actin is visualized using phalloidin 633 (*blue*). (**d**) Restoration of the staircase shape of a stereociliary bundle of a homozygous *Myo15ash2* vestibular hair cell 67 h after GFP-Myo15a transfection. Note the short length of stereocilia of non-transfected neighboring *Myo15ash2* hair cells. (**e**) Restoration of the staircase shape of the stereociliary bundle in a homozygous *Whrnwi* vestibular hair cell 48 h after transfection with GFP-Whrn. (**f**) Exogenous GFP-myosin 15a (*green*) recruits endogenous whirlin stained with anti-whirlin HL5136 antibody (*blue*) to stereociliary tips of a *Myo15ash2* transfected vestibular hair cell. Note that there is no anti-whirlin immunoreactivity in the stereocilia of neighboring non-transfected hair cells (*left*). (**g**) Rat vestibular hair cell stereociliary 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 stereociliary bundle of a non-transfected neighboring hair cell remains intact. (**h**) Degeneration of a stereociliary bundle transfected with GFP-myosin 15a (GFP-Myo15a), in a mouse vestibular hair cell 26 h post-transfection. There is an enormous accumulation of GFP-myosin 15a at the tips of fused stereocilia. (**i**) Accumulation of variable amount of GFP-myosin 15a at the tips of stereocilia of two transfected mouse vestibular hair cells from the same sensory epithelium explant 45 h post-transfection. (**j**, **k**) Different patterns of GFP-β-actin distribution in stereocilia of simultaneously transfected mouse vestibular hair cells from the same explant 72 h post-transfection. (**j**) GFP-β-actin is mostly at the tips of stereocilia, whereas, (**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** and **13**). Vortex for 10–15 s, then sonicate the tube for 30 s 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 s) 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 s) 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. 1a). Insert the tubing into the Tubing Prep Station, leaving about 10 cm of the tube sticking out on the right-hand side (Fig. 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 1000 × *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 1000–2000 × *g* at room temperature for 10 s, aspirate supernatant as in Subheading 3.2, **step 8**, 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 Subheading 3.2, **step 7**. 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 s 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* Subheading 2.2, **step 13**) 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* Subheading 3.2, **step 10**) 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. 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. 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* Subheading 3.2, **step 11**) 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 s. 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 s, allowing the gold to uniformly smear on the inside surface of the tubing.
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 ~1.3 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* Subheading 2.2, **step 14**) with one capsule of drycap dehydrators (Ted Pella, Inc., Redding, CA) in each vial (*see* Subheading 2.2, **step 14**). 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 trial shots, discharge the Helios® Gene Gun two to three 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. 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. 1b, c). Immediately position the plastic ring, located at the end of the Helios® Gene Gun barrel (Fig. 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. 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 1–5** 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 1× 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 1× PBS for 5 min.
4. Permeabilize in 0.5 % Triton X-100 for 10–15 min (*see* **Note 17**).
5. Wash four times with 1× PBS for 5 min.
6. Incubate in blocking solution (2 % BSA and 5 % goat serum in 1× 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 1× PBS for 5 min.
9. Incubate simultaneously for 20 min at room temperature in secondary antibody diluted in blocking solution (*see* Subheading 2.4, **step 6**) and phalloidin conjugated to a particular fluorophore (Invitrogen) and diluted 1:100 in blocking solution (*see* Subheading 2.4, **step 7**) (*see* **Note 18**).

10. Wash four times in 1× 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* Subheading 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 Gold Antifade Mountant (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 the next day using a confocal microscope equipped with a 63× or 100×, 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 15a and DsRed- or GFP-whirlin into hair cells of inner ear sensory epithelial explants obtained from wild-type and mutant mice [16, 18, 19]. In wild-type hair cells, unconventional motor protein myosin 15a and PDZ domain containing protein whirlin localize together at the tips of stereocilia [18, 19]. 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 15a 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 [18]. These data complemented observations of the endogenous myosin 15a and whirlin at the tips of stereocilia as revealed by immunofluorescence [16] and, most importantly, demonstrated that GFP and DsRed epitope tags do not interfere with proper targeting or function of these two proteins (Fig. 2a–c). Moreover, overexpression of GFP-myosin 15a in stereocilia of wild-type hair cells causes distention of stereocilia tips due to an accumulation of an excessive amount of GFP-myosin 15a [18]. No over-elongation of stereocilia of wild-type hair cells due to overexpression of myosin 15a was observed [16, 18].

Myosin 15a mutant (*Myo15ash2*) and whirlin mutant (*Whrnwi*) mice are deaf and have defective hair cell stereocilia that fail to elongate to a normal length due to mutations of these genes [55–57], and thus remain abnormally short [16, 18, 19, 55–58]. Gene Gun-mediated transfections of wild-type GFP-myosin 15a 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 stereociliary bundles (Fig. 2d–e). Moreover, using Gene Gun-mediated transfection of domain-deletion constructs of

myosin 15a and whirlin cDNAs into *Myo15ash2* and *Whrnwi* hair cells, we found that these two proteins interact in vivo through the C-terminal PDZ ligand of myosin 15a and the third PDZ domain of whirlin [18]. This interaction allows myosin 15a 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 15a "reawakens" the elongation process in the abnormally short *Myo15ash2* hair cell stereocilia by recruiting endogenous whirlin to stereociliary tips (Fig. 2f and [18]).

3.5.1 Advantages

Using lipofection, viral-mediated transfection, electroporation, or injectoporation, a transfected cell occasionally receives a very small amount of a cDNA expression construct encoding a fluorescently tagged protein. In this case, the amount of synthesized fluorescently tagged protein might be below the threshold of detection by fluorescence microscopy and some transfected cells might be indistinguishable from and mistaken for an 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 Helios® Gene Gun transfection is that you can usually find a gold particle inside the body of a transfected cell. This gold particle, marking a transfected cell, allows an investigator to distinguish a likely transfected cell from adjacent non-transfected cells that lack gold particles and can serve as non-transfected controls. Thus, in Helios® Gene Gun transfected epithelia, reliable control non-transfected cells of the same type can be identified in close proximity with transfected cell. Moreover, in our experiments (*see* Subheading 3.5), stereocilia from *Myo15ash2* and *Whrnwi* inner ear explants undergo elongation only when transfected with wild-type GFP-tagged myosin 15a or whirlin, correspondingly. In contrast, stereocilia bundles of non-transfected hair cells from the same explant remain short, because they are lacking functional myosin 15a (Fig. 2d, f) or whirlin (Fig. 2e). Thus, Gene Gun transfections provide an 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 experiments using Helios® Gene Gun-mediated transfection reveal the functional significance of specific proteins to key developmental events, as demonstrated by the importance of myosin 15a and whirlin interaction to the differential elongation of stereocilia during hair bundle morphogenesis [18].

3.5.2 Disadvantages

There are limitations to using Helios® Gene Gun-mediated transfections of cultured inner ear sensory epithelia. First, stereocilia are sensitive to mechanical disturbances and are easily damaged by an excessive pulse pressure of helium gas, triggering stereocilia degeneration. An early sign of degeneration is an abnormal hair bundle

shape (Fig. 2g–h). Stereocilia may show unrestrained elongation and/or fusion and some proteins in stereocilia become mislocalized, including GFP-tagged proteins introduced via cDNA constructs (Fig. 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, fused, splayed, or otherwise disorganized stereociliary bundle of a transfected *Myo15ash2* hair cell may likely result from degeneration rather than from overexpression of GFP-myosin 15a. Moreover, one can observe a single degenerating over-elongated stereocilium within a short *Myo15ash2* stereociliary bundle, even in non-transfected *Myo15ash2* hair cells that lack functional myosin 15a [16, 18]. Therefore, the presence of abnormal hair bundles with over-elongated stereocilia should not be interpreted as a result of GFP-myosin 15a overexpression in transfected wild-type hair cells (e.g., Fig. 2g–h). Rather, in both transfected and non-transfected *Myo15ash2* hair cells, over-elongation of stereocilia can be interpreted as a consequence of degeneration caused by either mechanical damage due to helium gas pressure, the culture conditions for a prolonged period of time, or the *Myo15ash2* mutation itself. To avoid excessive mechanical damage to stereocilia, the pressure of helium gas should be carefully adjusted for each particular Gene Gun and experimental setup. In our hands, a pressure of 110 psi was used successfully to obtain healthy-looking transfected cells.

Third, different amounts of plasmid DNA on the gold microcarriers may affect the outcome. While only one gold particle may penetrate a hair cell for most transfections, transfected hair cells may show different levels of GFP-tagged protein expression at a specific point in time. Figure 2i shows different amount of GFP-myosin 15a accumulation at the tips of hair cell stereocilia from the same explant 45 h post-transfection. A second example is GFP-β-actin, which first appears at the tips of stereocilia and then supposedly incorporates into actin filaments. Moreover, according to new data obtained from transfected live hair cell time-lapse images, GFP-β-actin may elongate some or all of the stereocilia from the tips, with elongated portions of stereocilia highlighted in green [49]. Hair cells simultaneously transfected with GFP-β-actin in the same explant may show different distribution patterns of newly incorporated GFP-β-actin within stereocilia. Similar variations in GFP-β-actin pattern of distribution in transfected hair cells from the same explant were described for viral infection/delivery to hair cells [54]. Figure 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. 2j), while GFP-β-actin highlights most of the stereociliary length of another hair cell (Fig. 2k). The time of appearance and the amount of GFP-tagged protein visualized in a transfected cell is 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 variations and 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 skepticism. Rigorous evaluation and accurate interpretation of the data from Gene Gun-mediated transfections may require, for example, good antibody to show that endogenous localization of this protein corresponds to the localization of GFP-tagged protein. Time-lapse imaging of transfected cells and the genetic analyses of the phenotype of relevant mutant mice are also useful.

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 optimize the operating parameters for their particular model. O'Brien and Lummis [42] developed a modified barrel for the Bio-Rad hand-held Helios® Gene Gun, which 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). Zhao and co-authors [22] reported another protocol improvement for the Gene Gun transfection. Although the efficiency was similar for both original and improved Gene Gun transfections, less damage to stereociliary bundles was claimed with this new procedure. The main differences from the standard procedure were a transbasilar-membrane approach for gold particle bombardment and a shortened version of focusing nozzle originally designed by O'Brien and Lumis [42].

   When transfection conditions were optimized for our standard protocol, taking into account all of the above-mentioned variables for our application and using the original Bio-Rad Helios® hand-held Gene Gun, we obtain good efficiency of transfection of both organ of Corti and vestibular sensory epithelia as illustrated in Fig. 3a. Using Clic5-GFP construct, many non-sensory cells as well as 14 hair cells have been transfected in the apical piece of the organ of Corti, but only the most apical portion of this piece is shown on the right panel of

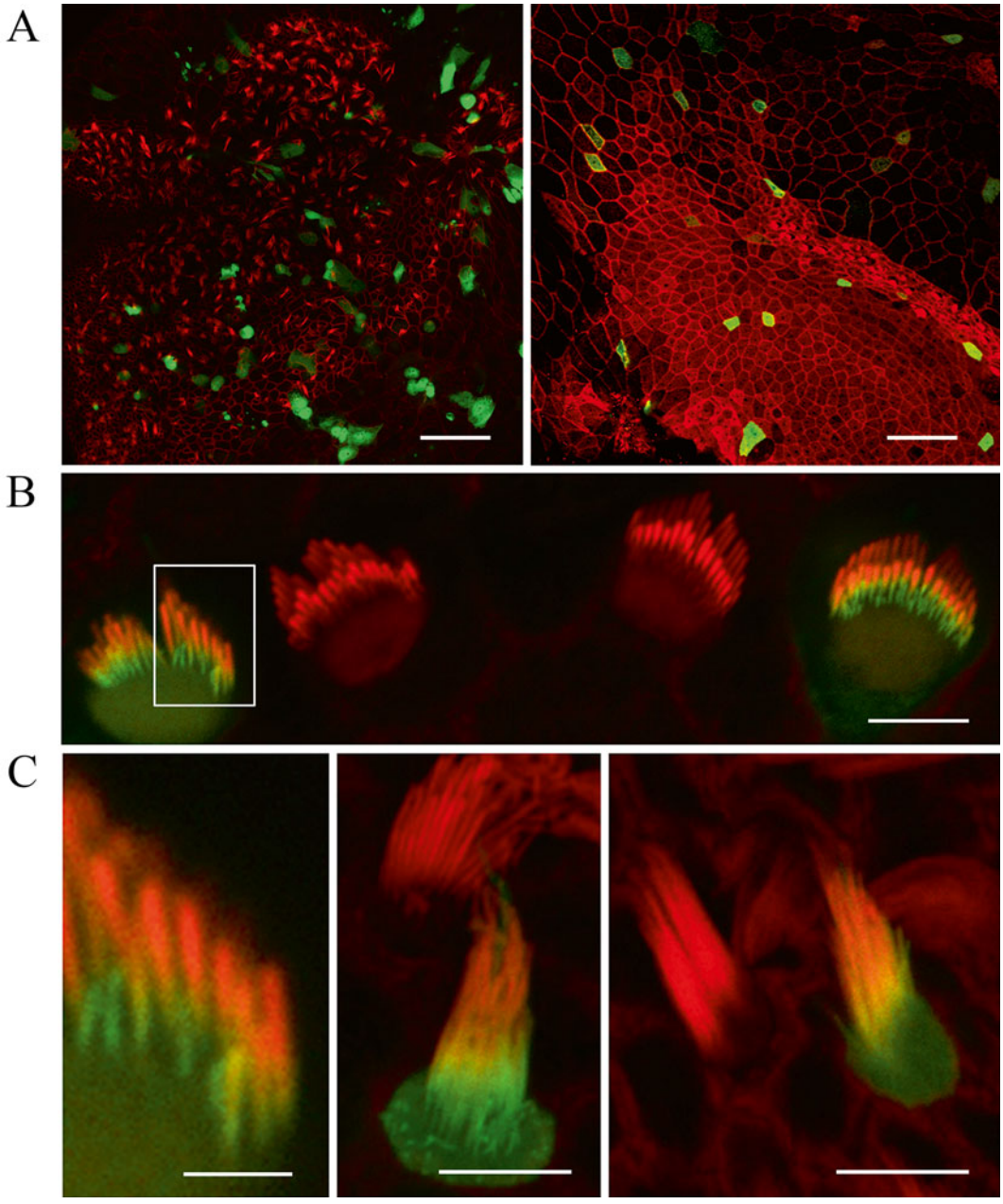

Fig. 3 Gene Gun-mediated Clic5-GFP transfection of the wild-type mouse inner ear sensory epithelia. (**a**) An overview image of the transfected sensory epithelium of the saccule (*left*) and the organ of Corti (*right*) taken with a 40× objective. The transfected cells are depicted in green. (**b**) Two non-transfected inner hair cells and two neighboring Clic5-GFP transfected inner hair cells from a P4 C57Bl/6J mouse organ of Corti. Clic5-GFP (*green*) is concentrated at the base of hair cell stereocilia of all rows. Boxed area in (b) is enlarged in (**c**, *left panel*). (**c**) Middle and right panels show transfected vestibular hair cells next to the non-transfected neighboring hair cells from a P4 C57Bl/6J saccule. Cytoskeletal actin is visualized by rhodamine-phalloidin (*red*) in all panels. Scale bars: in (**a**) and (**b**) 20 μm, in (**b**) and (**c**) 5 μm, except in the *left panel* in (**c**) is 1 μm

Fig. 3a. Close-up view of transfected hair cells shows that Clic5-GFP targets the tapered region of IHC stereocilia and highlights the entire length of the thinner underdeveloped third row stereocilia (Fig. 3b, c, left panel). In vestibular hair cells of the saccule, Clic5-GFP also targeted near the base of stereocilia (Fig. 3c, middle and right panel), mimicking its reported endogenous localization [51, 59, 60].

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 Institutional Animal Care and Use Committee.
4. All microdissections of the inner ear sensory epithelia should be carried out under sterile conditions using autoclaved instruments and preferably, in a laminar flow hood.
5. Glass-bottom 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 be used.
7. This microdissection curette allows transfer of sensory epithelia pieces submerged in a limited volume of L-15 media. Pieces of the sensory epithelia can also be transferred using a glass Pasteur pipette (*see* Subheading 2.4, **step 8**) attached to a pipette holder (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 the specimen along 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) with the tip of the glass pipette, when you want to release your specimen into DMEM. This way the specimen will be transferred to 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. To use a different vector with your cDNA of interest you can purchase a Quantum Prep Plasmid Miniprep Kit (100 preps, Bio-Rad) or Qiagen's QIAfilter plasmid midi kits (Qiagen, Valencia, CA), to prepare plasmid DNA of high purity suitable for Helios® Gene Gun transfection. It is advisable to use endotoxin-free kits to prepare high-purity plasmid DNA. 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 (>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 a poor transfection outcome as well as gold particle clustering. After purification, DNA should be diluted to 1 mg/mL in molecular biology grade water (*see* also [42]).

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. For example, nanoparticles are used in the improved method of neuron transfection in the cultured brain slices [61–63]. However, whether nanoparticles under similar conditions penetrate the cuticular plate of hair cells needs further investigation. Tungsten particles are less expensive, but can oxidize and may be toxic to the cells. It is not recommended to use tungsten particles instead of gold [64].
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. The crystallized PVP is hygroscopic. Store it in a tightly closed vial in a desiccator at room temperature.
13. Spermidine solution should be sterile-filtered using a 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 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 [42]. 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, as well as in the previous version of this chapter published in 2009 [65], sonication is omitted only in steps when DNA is added to gold particles to avoid the potential destructive effects of sonication on DNA.
15. The use of a BioRad diffusion screen reduces the damage to the inner ear sensory epithelia and especially to hair cell stereociliary 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 will build up on the center of the diffusion screen, which may become gold colored. Use a dedicated diffusion screen for each plasmid DNA to avoid cross-contamination. A dedicated diffusion screen for a particular plasmid DNA can be reused many times. It does not

require frequent autoclaving. However, it is necessary to clean diffusion screens before using them 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 to generate steam. Some autoclaves include a detergent in the water and this situation should be avoided.

16. 1× PBS containing Ca^{2+} and Mg^{2+} is used if the presence is required of these ions.
17. Triton X-100 is a viscous solution. It is useful to prepare a 1 % stock solution by adding 500 μL of Triton X-100 to 49.5 mL of 1× PBS. Before introducing Triton X-100 into a 1 mL pipette tip, cut off about 1 cm of the tip to make a wider opening. This help in drawing the 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.
19. ProLong Gold Antifade Mountant can be stored at room temperature, but have to be tightly closed when not in 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). 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 cultures.
22. Alternatively, to store cartridges you can use tightly closed 15 mL conical tubes, each with one capsule of dehydrator as described in Subheading 2.2, **step 14**. Some preparations of plasmid DNA coated onto gold particles were used successfully for transfections after more than 2 years of storage with proper desiccation.
23. To prevent culture contamination, wipe the plastic ring of the end of a barrel (Fig. 1c, black arrow) with 70 % ethanol after each shot. It is advisable to wear ear protection (earmuffs or earplugs) when firing the Gene Gun.

24. It is important to remove the 1× 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 the nail polish dry before using the slide under a confocal microscope. For long-term storage up to a few months, store slides in the slide boxes at 4 °C with several capsules of desiccant (Ted Pella, Inc.).

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