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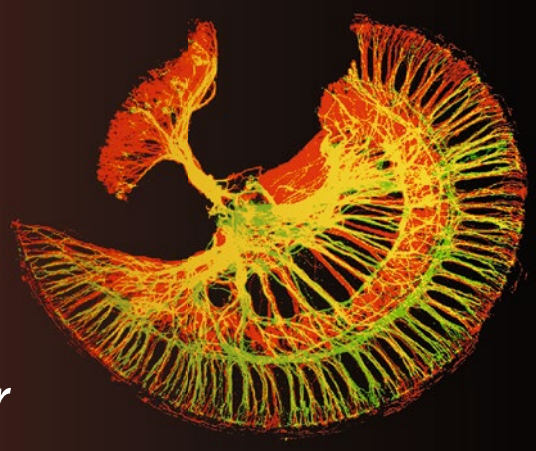

Bernd Sokolowski *Editor*

Auditory and Vestibular Research

Methods and Protocols

Second Edition

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Humana Press

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Auditory and Vestibular Research

Methods and Protocols

Edited by

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Cover illustration: Cover photo shows a confocal image (Leica SP5) of afferents (Neurovue Orange, red) and efferents (Neurovue Maroon, green) to the basal turn of the organ of Corti in a postnatal mouse. Image provided by B. Fritzsch.

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Preface

Seven years have passed since the last edition of *Auditory and Vestibular Research, Methods and Protocols.* During this period of time, technological advances and research findings in the field continued at a rapid pace. Thus, no single edition could encompass all the different types of experiments and protocols conducted in the auditory and vestibular fields. With this, the second edition of *Auditory and Vestibular Research*, *Methods and Protocols*, we expand the previous volume from three to seven major sections. Here, we introduce new protocols that encompass cell culture, tissue engineering, nanotechnology, high-throughput screening, and physiology. The section on physiology alone covers techniques that include optical coherence tomography, patch clamping, and photostimulation of caged neurotransmitters. While the first edition explored the nuances of DNA/RNA and protein protocols, the second edition further expounds on these techniques with new chapters and updates. The imaging section in this edition elucidates traditional areas of fluorescence microscopy, including how to build your own fluorescence microscope, but also contains newer techniques that allow the scanning of live stereocilia at nanoscale resolution and large-scale mapping of the brain using electron microscopy (EM). As in the first edition, the present overview provides a perspective of basic research with both mammalian and non-mammalian animal models. The chapters in Part I focus on RNA delivery and extraction, while those in Part II bring updates to protein protocols such as the yeast two-hybrid system and plasmon resonance, while adding new chapters on protein stoichiometry and colocalization. Part III covers various microscopy techniques, including confocal fluorescence, hopping probe ion conductance, and EM to study connectomics. Part IV describes culture protocols such as those used in organ culture, quantifying neurite behavior, and tissue engineering using umbilical cord cells. Part V focuses on nanotechnology with a general overview of nanoparticle-based delivery in hearing disorders and, more specifically, nanotechnology in membrane electromechanics. Part VI entails a description of inner ear cell sorting techniques and high-throughput chemical screens. Finally, Part VII contains seven chapters describing physiological techniques that measure responses beginning with the basilar membrane, continuing with hair cells, their stereocilia, and spiral ganglion cells, and ending with central auditory circuits. The techniques described herein will be useful to scientists in other fields, especially where tissues are scarce and where a comparative approach is useful in discovering the causes of human disorders.

Tampa, FL, USA ***Bernd Sokolowski***

Acknowledgements

I would like to give my heartfelt thanks to all of the authors who contributed to the two volumes of *Auditory and Vestibular Research, Methods and Protocols*. A special thanks is extended also to the series editor, John Walker, and to the staff of Springer Verlag for their attention to quality in publishing.

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Part I

RNA Delivery and Extraction Protocols

Chapter 1

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

Inna A. Belyantseva

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).

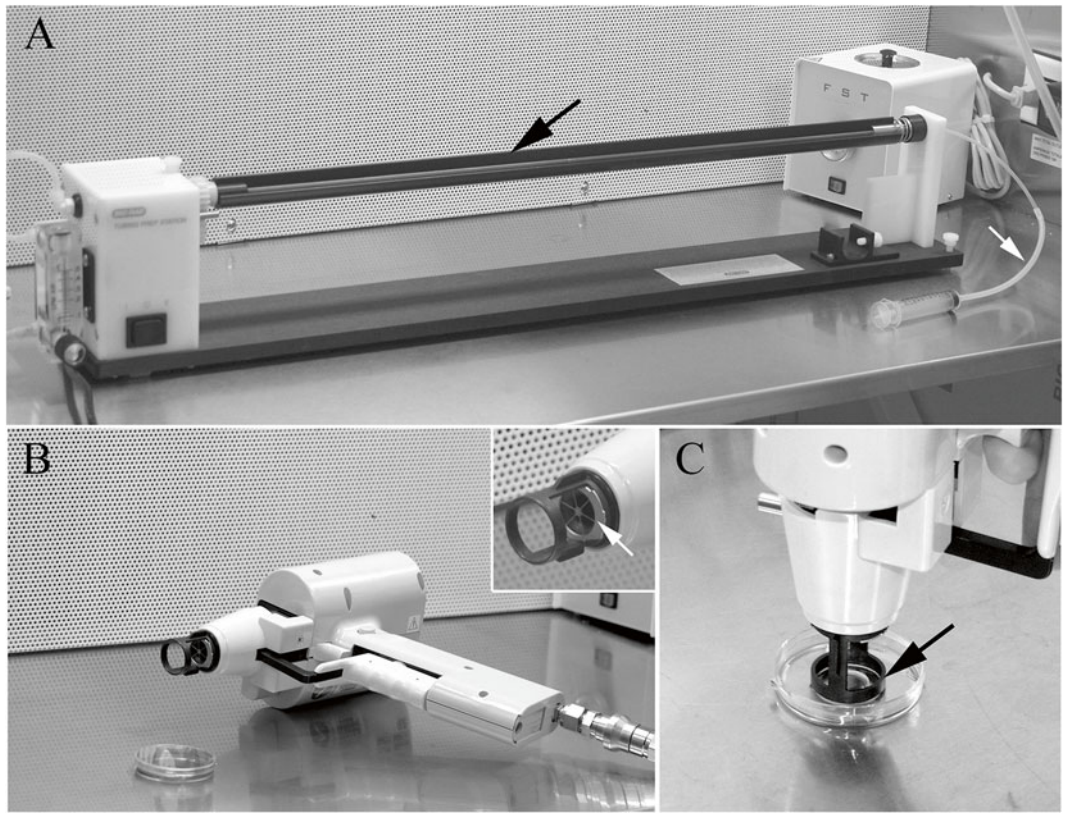

Fig. 1 Bio-Rad Helios® Gene Gun and Tubing Prep Station. (**a**) Tubing Prep Station with Tefzel tubing inserted into the tubing support cylinder (*black arrow*). The right end (~15 cm) of the Tefzel tubing is sticking out and is connected to the 10 cc syringe with adaptor tubing (*white arrow*). (**b**)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