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Stable Transformation of Plant Cells by Particle Bombardment/Biolistics

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Summary

Particle bombardment, or biolistics, is a commonly used method for genetic transformation of plants and other organisms. Millions of DNA-coated metal particles are shot at target cells or tissues using a biolistic device or gene gun. The DNA elutes off the particles that lodge inside the cells, and a portion may be stably incorporated in the host chromosomes. A protocol for the generation of transgenic grapevines via biolistic transformation of embryogenic cell suspension cultures is detailed in this chapter. In a typical experiment, transient gene expression averaged nearly 8000 "hits" per bombarded plate. Five months after bombardment, there were nearly five putative transgenic embryos per bombarded plate. About half of the embryos were regenerated into confirmed transgenic plants. The basic bombardment procedures described are applicable to a wide range of plant genotypes, especially those for which embryogenic cell cultures are available. All users of particle bombardment technology will find numerous useful tips to maximize the success of transformation.

Key Words: Ballistics; biolistic; biotechnology; embryogenic cells; gene gun; genetic engineering; grapevine; microcarrier; microparticle bombardment; microprojectile bombardment; particle acceleration; particle bombardment; particle gun; plant transformation; *Vitis*.

1. Introduction

Particle bombardment employs high-velocity microprojectiles to deliver substances into cells and tissues. For genetic transformation, DNA is coated onto the surface of micron-sized tungsten or gold particles by precipitation with calcium chloride and spermidine. Once inside the cells, the DNA elutes off the particles. If the foreign DNA reaches the nucleus, then transient expres-

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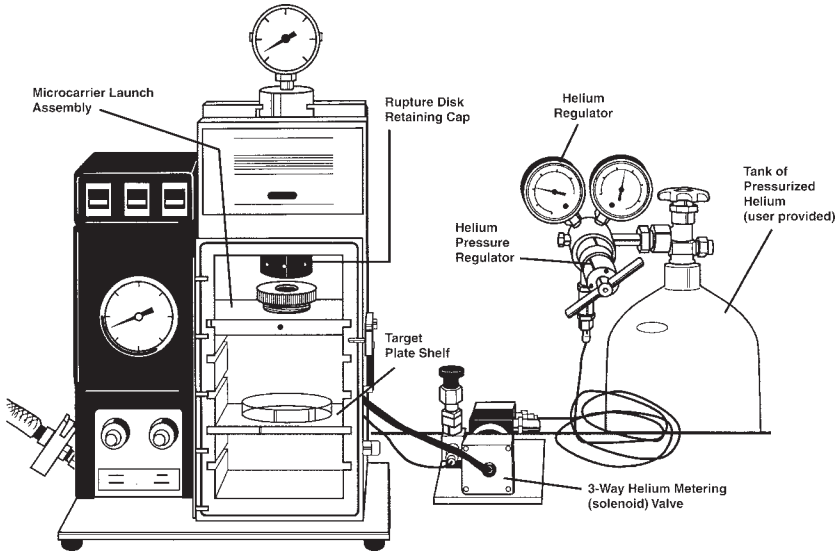

Fig. 1. Components of the Biolistic® PDS-1000/He particle delivery system. (Drawing courtesy of Bio-Rad Laboratories, Hercules, CA.)

sion will likely result and the transgene may be stably incorporated into host chromosomes. Sanford and colleagues at Cornell University developed the original bombardment concept ***(1,2)*** and coined the term "biolistics" (short for "biological ballistics") for both the process and device. "Biolistics" is a registered trademark of E. I. du Pont de Nemours and Co. and has been used to market the devices now sold by Bio-Rad Laboratories, Hercules, CA. However, as there are several homemade "gene guns" or "particle guns," the process often is called by other names such as microprojectile bombardment, particle bombardment, particle acceleration, or ballistics.

The most widely used device for plant transformation is the Biolistic® PDS-1000/He Particle Delivery System ***(3)*** marketed by Bio-Rad Laboratories (**Fig. 1**). The system employs high-pressure helium released by a rupture disk to propel a macrocarrier sheet loaded with millions of DNA-coated metal particles (microcarriers) toward target cells (**Fig. 2**). A stopping screen halts the macrocarrier, and the microcarriers continue toward the target and penetrate the cells.

Because of its physical nature and simple methodology, the biolistic process can be used to deliver substances into a wide range of intact cells and tissues from a diversity of organisms. In plant research, the major applications have been transient gene expression studies, production of genetically transformed

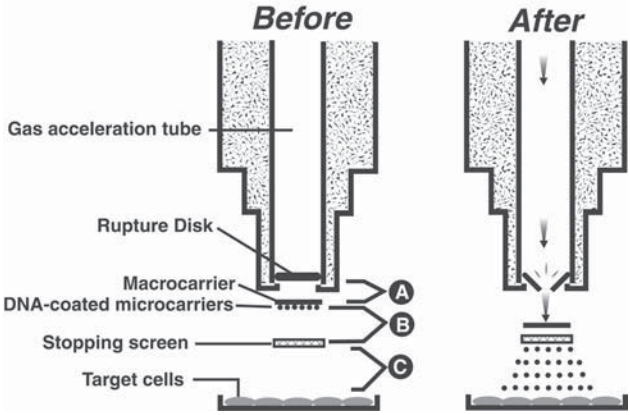

Fig. 2. The Biolistic® bombardment process. The gas acceleration tube is filled with helium gas until the maximum pressure of the rupture disk is reached. When the disk ruptures, the ensuing helium shock wave launches a plastic macrocarrier onto which the DNA-coated microcarriers have been dried. The macrocarrier flies downward until it impacts a stopping screen. On impact, the macrocarrier is retained by the stopping screen, while the microcarriers are launched and continue downward at high velocity until they impact and penetrate the target cells.

The velocity of the macrocarriers is dependent on the helium pressure in the gas acceleration tube, the distance from the rupture disk to the macrocarrier (gap distance) (A), the macrocarrier travel distance to the stopping screen (B), the distance between the stopping screen and target cells (C), and the amount of vacuum in the bombardment chamber. (Drawing courtesy of Bio-Rad Laboratories, Hercules, CA.)

plants, and inoculation of plants with viral pathogens ***(2,4,5)***. Many "firsts" were achieved through the application of biolistic technology including chloroplast and mitochondria transformation, as well as nuclear transformation of important monocot species such as wheat, corn, and rice ***(2)***. Although other technologies have since been proven in these arenas, Sanford in the year 2000 ***(2)***, stated the following: "I believe it is accurate to say that most of the presently grown transgenic crop acreage in the entire world was created through the use of the biolistic process—having been originally transformed with the gene gun."

As with any plant transformation method, several parameters need to be optimized for the process to be maximally effective. With biolistics, the parameters can be grouped as physical, biological, and environmental ***(4–7)***. Physical parameters include the composition and size of the microcarriers, the attachment of DNA to the microcarriers, and several instrument parameters.

During development of the PDS-1000/He, instrument settings were varied over a wide range and tested with numerous organisms ***(7,8)***. A vacuum of 28.0 in Hg (94.8 kPa), a helium pressure of about 1100 psi (7584.2 kPa), a gap distance of 6.5–10.0 mm, and a macrocarrier travel distance of 6.0–10.0 mm are near optimal for most plant transformation applications. Gold particles in the range of 0.7–1.0 μm mean diameter generally result in the highest rates of stable transformation, but the less expensive, more heterogeneous tungsten particles are also widely used. Consistent coating of DNA to the particles and spread of the particles onto the macrocarrier are critical, and proficiency develops with practice.

The first biological parameter to consider is a gene construct in the form of a circular or linear plasmid or a linear expression cassette (promoter–gene–terminator). It is important to match the promoter and/or other regulatory sequences with the plant tissue, so that the gene will be expressed at desired levels. Other biological parameters include tissue type, cell size, cell culture age, mitotic stage, general cellular health, target tolerance of vacuum, cell density, and cell turgor pressure. The physiological status of the target influences receptivity to foreign DNA delivery and susceptibility to injury that may adversely affect the outcome of the transformation process. For recovery of transgenic plants, it is very important to target cells that are competent for both transformation and regeneration. Furthermore, the ability of bombarded cells to regenerate plants depends on the type and concentration of the selection agent. In some cases, it is best to start with a low concentration of the selective agent and increase it after 2 or 4 wk of cell culture.

Environmental factors such as temperature; humidity; and light intensity, quality, and duration have a direct effect on tissue physiology and thus transformation success ***(6)***. In addition, some explants may require a "healing" period after bombardment under special regimens of light, temperature, and humidity ***(6)***. Humidity also is important in microcarrier preparation and bombardment. High humidity can cause the microcarriers to clump and/or to bind irreversibly to the macrocarrier, thus reducing transformation rates. High humidity may also affect stocks of alcohol used during the DNA/microcarrier coating steps. Some researchers use cold temperatures while coating macrocarriers with DNA, whereas our laboratory uses room temperature. We are not aware of a published study on the effect of temperature on microcarrier coating or bombardment.

There has been much discussion over the advantages and disadvantages of the biolistic process as compared to *Agrobacterium* (*see* Chapter 2) for the production of transgenic plants. The physical nature of the biolistic process eliminates concerns about using another biological organism in the transformation process. In grapevines, there is often a hypersensitive response to *Agrobacterium* that causes plant cell death ***(9)***. Biolistics obviates both the

need to kill *Agrobacterium* after transformation and the occurrence of false positives arising from growth of *Agrobacterium* in the host tissues. Operation of the biolistic device is easy and there are only a few instrument parameters to adjust. Because the Biolistic® PDS-1000/He unit is commercially available, the user benefits from convenience, ease of use, technical support, and standardization with other labs. Furthermore, plasmid construction is often simplified and cotransformation with multiple transgenes ***(10)*** is routine, because plasmid DNA is simply mixed together before coating onto the microcarriers. The use of linear expression cassettes (also called clean gene technology) eliminates the chance that extraneous plasmid backbone DNA will be inserted into the target as can happen with whole plasmids or *Agrobacterium* ***(11)***. Biolistics is the method of choice for the study of transient gene expression and for plastid transformation ***(5)***. Furthermore, biolistics is the only successful method of transformation currently available for certain genotypes ***(5)***.

Some disadvantages of biolistics are that the transformation efficiency may be lower than with *Agrobacterium* and the device and consumables are costly. Many researchers have strayed from biolistics because of the tendency for complex integration patterns and multiple copy insertions that could cause gene silencing. Some laboratories have overcome this problem by reducing the quantity of DNA loaded onto the microcarriers and/or by use of linear cassettes ***(11)***. Random integration is also a concern and is being addressed by several groups ***(5)***, the most promising being the use of the Cre–Lox system for targeted integration ***(12)***.

As many parameters need to be optimized for any transformation method, often the experience of the investigator and nearby colleagues determines which method is chosen. The user must weigh the advantages and disadvantages of the various methods available. Patents and licensing availability should also be considered. Particle bombardment technology is covered by several patents held by E. I. du Pont de Nemours and Co. and PowderJect Vaccines, Inc. Use of particle bombardment for commercial purposes may require a commercial license from the appropriate patent holder. There are also patents held by different companies for the use of particle bombardment for certain plant species such as *Zea mays*. Thus, patents rights must be investigated thoroughly. In comparison, patent rights for *Agrobacterium*-mediated transformation are less clear and are tied up in the legal system. Thus, obtaining a license for *Agrobacterium* is more difficult for those outside of the patent-holding companies.

Our laboratory has successfully employed biolistics to obtain transgenic grapevine plants. This chapter details a protocol for transformation of *Vitis vinifera* L. 'Chardonnay' embryogenic suspension cultures in which numerous transformation parameters have been optimized ***(13)***. Bombardment with gold particles coated with plasmid pBI426 (double CaMV 35S promoter,

Alfalfa mosaic virus (AMV) leader sequence, *uidA* gene, nos terminator) resulted in an average of 7883 ± 1928 β-glucuronidase (GUS) positive blue spots per Petri plate at 2 d and 46 ± 32 at 95 d. A total of 447 embryos were harvested from 84 bombarded plates on selection medium within 5 mo after cobombardment with two separate plasmids. This represents more than 5 putative transgenic embryos per bombarded plate. From those, 242 plants were regenerated, which corresponds to a 54% rate of conversion of embryos to regenerated plants. The cotransformation frequency of genes on different plasmids was in the range of 50% in the group of regenerated plants ***(13)***. The basic cell handling and bombardment procedures have been used for numerous other genotypes ***(7)***; however, the media and environmental conditions for cell growth, transformant selection, and plant regeneration must be optimized for each.

2. Materials

All reagents should be tissue culture or molecular biology grade.

2.1. Culture and Preparation of Plant Cells

1. Plant material: embryogenic *Vitis vinifera* L. 'Chardonnay' cell suspension cultures ***(13)*** (*see* **Note 1**).
2. Medium for cell suspension cultures: (GM + NOA medium ***[14]***): Murashige and Skoog (MS) ***(15)*** basal medium (macro- and microelements, vitamins, and inositol) with 18 g/L of maltose hydrate, 4.6 g/L of glycerol, and 5 μ*M* β-napthoxyacetic acid (NOA). Adjust pH to 5.8 with KOH before autoclaving. To prepare 100 mL of a 1 m*M* NOA stock solution, dissolve 20.2 mg of NOA in 2 mL of 1 *M* KOH. Stir briefly and add 90 mL of Type I water. Continue stirring for 1 h. Bring to final volume and filter sterilize. Store at 4°C; stock is good for 1 yr. Use 5 mL of stock per liter of media.
3. 250-, 500-, and 1000-mL Erlenmeyer flasks, capped with aluminum foil and autoclaved.
4. Double-screen mesh (1.1 mm^2 pore size) in a polypropylene funnel to filter cell suspensions, autoclaved.
5. Disposable 10- and 25-mL plastic pipets, cotton-plugged, sterile.
6. Compound microscope, glass slides, and cover slips.
7. Magnetic stir plate and autoclaved stir bar.
8. Graduated 12- or 15-mL conical centrifuge tube.
9. 100-mL media bottle with screw cap lid, autoclaved.
10. 1-mL sterile polyethylene transfer pipet.
11. Büchner funnel (8 cm in diameter, autoclaved), size arm flask (1 L, autoclaved), and vacuum source.
12. 7-cm diameter Whatman no. 2 filter papers, autoclaved.
13. Bombardment medium (1/2 MS-HF [hormone-free] medium with osmotica [*see* **Note 2**]): MS medium with half-strength macro- and microelements, full-strength vitamins and inositol, 30 g/L of sucrose, 0.125 *M* mannitol, 0.125 *M* sorbitol, and

2.5 g/L of Phytagel (Sigma, St. Louis, MD). Adjust pH to 5.8 with KOH before autoclaving. Dispense in 10-mL aliquots on top of a sterile, circular filter paper (S&S Sharkskin, 9 cm in diameter, VWR International, South Plainfield, NJ, cat. no. 28314-028) that is contained in a 100 × 15 mm Petri plate. The filter paper should have a small tab of tape attached (homemade) so that once the medium is solidified; forceps can be used to pick up the whole unit by the tab. The sterile medium can be stored in sterile plastic bags at room temperature for 1 mo.

14. Sterile forceps.

2.2. Preparation of DNA-Coated Microcarriers

2.2.1. Sterilization of Macrocarriers and Holders

1. Macrocarriers for biolistic device (Bio-Rad).
2. Macrocarrier holders (Bio-Rad).
3. 70 and 95% ethanol.
4. Glass beaker and glass Petri plate (autoclaved).
5. Sterile Kimwipes or paper towels.
6. Sterile forceps with fine point tips (curved tips work well).
7. Desiccant in glass Petri dishes. A sterile filter paper or inverted plastic Petri plate with holes (homemade) should be placed over the desiccant to provide a stable, dust-free platform for loading DNA-coated particles onto the macrocarriers. We use Drierite brand desiccant, which changes from blue to pink as it absorbs water. Bake at 180°C for approx 4 h to restore blue color and desiccating ability.

2.2.2 Sterilization of Gold Particles

1. Microcarriers: gold particles, 0.75 µm in diameter (Analytical Scientific Instruments, El Sobrado, CA) (*see* **Note 3**).
2. Small glass vial or tube (1–3 mL).
3. Oven that will reach 180°C.
4. 500-µL micropipettor and tips.
5. Isopropanol, HPLC grade.
6. 1.5-mL microcentrifuge tubes, autoclaved, Treff Lab, Degersheim, Switzerland (cat. no. 96.7246.9.02) (*see* **Note 4**).
7. Sterile type I water.
8. Glycerol (50% v/v): Mix glycerol 1:1 with type I water and autoclave.

2.2.3 Coating Gold Particles With DNA

1. Micropipettors and tips (5- to 500-µL range).
2. 1.5-mL microcentrifuge tubes, autoclaved, Treff brand (*see* **item 6** in **Subheading 2.2.2.**).
3. Plasmid DNA at 1 µg/µL in sterile TE buffer (1 m*M* Tris-HCl, pH 7.8, 0.1 m*M* disodium ethylenediaminetetraacetic acid [EDTA]) (*see* **Note 5**).
4. 2.5 *M* $CaCl_2$, filter-sterilized: To make 50 mL, dissolve 18.38 g of calcium chloride dihydrate in type I water. Filter sterilize and store at 4°C in small aliquots.

5. 0.1 *M* spermidine free base, filter-sterilized. Solid spermidine is very hygroscopic. Therefore, take a 1-g unopened bottle of spermidine free base (Sigma cat. no. S-0266), add 1 mL of type I water, adjust the volume to 68.9 mL, vortex to mix thoroughly, filter sterilize and store at –20°C in 1.2-mL aliquots in 1.5-mL microcentrifuge tubes with screw-cap lids. The stock is good for 1 mo (*see* **Note 6**). Discard individual tubes after first use.
6. Continuous vortex mixer such as the Vortex Genie-2 Mixer with 15.2-cm platform head (cat. no. 58815-178 and 58815-214; VWR, International, South Plainfield, NJ).
7. HPLC grade isopropanol.
8. Ultrasonic water bath cleaner (Model B1200R-1; Branson Ultrasonics Corporation, Danbury, CT, or similar unit).

2.3. Bombardment

1. Biolistic® PDS-1000/He Instrument (Bio-Rad).
2. Helium gas cylinder; high pressure (2400–2600 psi [16,547.4–17,926.4 kPa]); grade 4.5 or 5.0 (99.995% or higher purity).
3. Vacuum pump; oil-filled rotary vane, with a pumping speed of 90–150 L/min (3–5 ft^3/min).
4. Rupture disks (1100 psi [7,584.2 kPa], Bio-Rad), sterilize with isopropanol (*see* **Note 7**).
5. Stopping screens (Bio-Rad), sterilize by autoclaving.
6. Safety glasses.
7. Hair net and latex gloves.
8. Opaque plastic box sterilized with 70% ethanol to store bombarded plates.

2.4. Postbombardment Reduction of Medium Osmoticum

1. Medium (1/2 MS-HF) without osmotica: MS medium with half-strength macro- and micro- elements, full-strength vitamins and inositol, 30 g/L of sucrose, and 2.5 g/L of Phytagel. Adjust pH to 5.8 with KOH and autoclave. Dispense in 10- and 20-mL aliquots into 100 × 15 mm Petri plates (*see* **Note 8**).
2. Sterile forceps.

2.5. Analysis of Transient and Long-Term GUS Expression

1. GUS histochemical staining solution: To prepare 200 mL, combine the following components: 150 mL of type I water, 0.744 g of EDTA, disodium salt, dihydrate, 1.76 g of sodium phosphate monobasic, 0.042 g of potassium ferrocyanide, and 0.2 mL of Triton X-100. Adjust the volume to 198 mL, and the pH to 7.0. Add 100 mg of 5-bromo 3-chloro 3-indolyl β-D-glucuronic acid (X-Gluc) that has been dissolved in 2 mL of dimethyl sulfoxide (DMSO). Filter sterilize and store at –20°C; stock is good indefinitely.
2. Sterile forceps.
3. Petri plates, 100 × 15 mm diameter, sterile.
4. Incubator, 37°C.

5. Stereomicroscope.
6. Plastic sheet with an imprinted grid (homemade).
7. Cell counter.

2.6. Embryo Selection, Germination, and Regeneration

1. Kanamycin monosulfate (Km) stock (25 mg/mL, pH 5.8, filter-sterilized). Prepare in type I water. Store at –20°C in small aliquots. Frozen stock is good indefinitely. Warm to add to autoclaved media (*see* **Subheading 2.6., item 2**) that has been cooled to 50–55°C.
2. Selective medium: 1/2 MS-HF medium with 30 g/L of sucrose, 3 g/L of activated charcoal, 7 g/L of Bacto-agar (Difco, Detroit, MI), and 10 or 15 mg/L of Km (added after autoclaving). Adjust pH to 5.8 with KOH and autoclave. Dispense in 20-mL aliquots into 100 × 15 mm Petri plates.
3. Embryo germination medium: 1/2 MS-HF (Km-free) with 30 g/L of sucrose, 3 g/L of activated charcoal, and 2.5 g/L of Phytagel. Adjust pH to 5.8 with KOH and autoclave. Dispense 20 mL per 100 × 15 mm Petri plate or 30 mL per baby food jar.
4. Plant growth medium: woody plant medium (WPM) ***(16)***, pH 5.8, with 20 g/L of sucrose and 2.5 g/L of Phytagel. Dispense 50 mL per Magenta GA7 vessel (Magenta Corp., Chicago, IL).
5. Parafilm (American National Can, Menasha, WI).
6. Venting Tape (Scotch brand no. 394; 3M Corporation, Minneapolis, MN).

3. Methods

Preparation for bombardment (*see* **Note 9**) should begin 6 d in advance (**Table 1**). All steps should be carried out in a laminar flow hood to avoid microbial contamination.

3.1. Culture and Preparation of Plant Cells

1. Maintain embryogenic suspension cells in GM+NOA medium in 250- or 500-mL Erlenmeyer flasks at 120 rpm, in the dark at 23 ± 1°C. Each week, the medium should be refreshed by removing and replacing one half of the spent medium with fresh medium using a sterile plastic 10- or 25-mL pipet (*see* **Note 10**). Cells should be poured through a funnel with sterile screen mesh (*see* **Subheading 2.1., item 4.**) to remove large clumps as needed.
2. Use cells for bombardment 4 d after subculture. The cell suspension culture should be checked immediately before use for microbial contamination by placing a sample on a glass slide with cover slip and observing it under a compound microscope. Fungal strands or bacteria can be easily recognized (*see* **Note 11**).
3. Pour all cells needed for bombardment through a sterile screen mesh in a funnel positioned over the mouth of a 1-L sterile Erlenmeyer flask. Add a sterile stir bar and place the flask on a magnetic stir plate (in a laminar flow hood). Turn the stir plate on a low setting to mix the cells.

Table 1
Flow Chart of Steps for Particle Bombardment Transformation

Time	Activity (in sequential steps)
	Week prior to bombardment
(–) 6 d	Sterilize supplies (Whatman and Sharkskin filter papers, funnels, flasks, water, etc.).
(–) 5 d	Prepare media needed for transformation procedure. GM+NOA suspension culture medium. 1/2 MS-HF bombardment medium with osmotica. 1/2 MS-HF medium without osmotica. 1/2 MS-HF selective medium.
(–) 4 d	Subculture or refresh medium of embryogenic cell suspensions.
	Week of bombardment
(–) 1 d	Set gene gun parameters (distances as described in **Fig. 2**). Weigh gold particles (microcarriers) and place in an oven overnight. Sterilize macrocarriers, holders and stopping screens. Assemble macrocarriers into holders.
Key d	Bombardment day (suggested day, Tuesday). Examine embryogenic cell suspension for contamination using a microscope. Prepare cells on filter paper for bombardment. Sterilize microcarriers. Coat microcarriers with DNA. Bombard cells. Incubate cells in the dark at 23 ± 1°C.
(+) 1 d	Transfer cells to medium without osmotica. First transfer approx 16 h after bombardment. Second transfer approx 24 h after bombardment.
(+) 2 d	Transfer cells to selective medium. Analysis of reporter gene (i.e., GUS assay) for transient expression.
(+) 3 d	Examine GUS-positive blue spots per filter paper.
	Postbombardment weeks
(+) 30 d	Transfer cells to fresh selective medium. Reporter gene assay for transient expression.
(+) 60 d	Check plates for development of embryos. Transfer embryos to germination medium. Transfer remaining cells to fresh selective medium. Reporter gene assay for long-term expression.
(+) 90 d	Items and procedure as in (+) 60 d. Transfer germinated embryos to plant growth medium.

4. To standardize cell density for bombardment, place a 10-mL sample of the cell suspension in a graduated 12- or 15-mL conical centrifuge tube and allow cells to settle for 15 min (30 min if cell suspension is very fine). Record the settled cell volume and discard the sample in the centrifuge tube. Adjust the density of the cell suspension in the flask to be used for bombardment to 0.2 mL of settled cell volume per 10-mL sample by adding or removing GM+NOA medium.
5. For each plate to be bombarded, place a sterile Whatman no. 2 filter paper in a Büchner funnel positioned on a 1-L side-arm flask. Using a sterile transfer pipet, remove 1 mL of GM+NOA medium from the small media bottle and place on the Whatman no. 2 filter paper to moisten it. While continuing to stir the cell culture, use a sterile 10-mL pipet to collect 5-mL of cells from the culture flask and then spread as a single layer onto the filter. Apply a slight vacuum to draw off excess liquid and to help spread the cells (*see* **Note 12**).
6. Transfer the filter paper with attached cells to bombardment medium using sterile forceps.

3.2. Preparation of DNA Coated Microcarriers

3.2.1 Sterilization of Macrocarriers and Holders

1. Place macrocarrier holders in a glass beaker and macrocarriers in a glass Petri dish. Fill containers with 70% ethanol and let stand for 15 min (*see* **Note 13**).
2. Remove the macrocarrier holders from the 70% ethanol with sterile forceps and place on sterile Kimwipes or paper towels in a laminar flow hood to dry.
3. Using sterile forceps, remove the macrocarriers from the 70% ethanol and dip them briefly in 95% ethanol. Place on sterile Kimwipes or paper towels in a laminar flow hood to dry.
4. Assemble macrocarriers into the holders using sterile forceps and place the units in glass Petri plates with desiccant.

3.2.2. Sterilization of Gold Particles

This protocol prepares enough particles for 60 shots.

1. Weigh 30 mg of gold particles and place into a glass vial.
2. Heat particles in an oven at 180°C for 12 h (*see* **Note 14**).
3. After cooling, add 0.5 mL of isopropanol and vortex-mix vigorously for 2 min.
4. Soak for 15 min, vortex-mix (1 min), and transfer into a 1.5-mL microcentrifuge tube.
5. Pellet by centrifugation at 13,000*g* for 1 min.
6. Carefully remove the supernatant with a pipet and discard.
7. Add 0.5 mL of sterile type I water and resuspend particles by vortex-mixing vigorously for 30 s.
8. Centrifuge for 1 min and discard the supernatant as before.
9. Repeat the water wash for a total of three times.
10. Resuspend particles in 0.5 mL of 50% (v/v) glycerol/type I water. Vortex-mix vigorously for 1 min. Particles are ready for use, or may be stored in 50-µL aliquots at 4°C for 1 mo.

3.2.3 Coating Gold Particles With DNA

This protocol is for six shots.

1. Vortex-mix gold particles vigorously for 2 min and dispense 50 µL of particles into a 1.5-mL microcentrifuge tube. Vortex-mix for 5 s before each subsequent particle dispensement and just prior to adding the DNA (*see* **Note 15**).
2. Add the following components sequentially and quickly to the tube:
   a. 5 µL of 1 µg/µL plasmid DNA (for cotransformation with two plasmids, use 2.5 µL of each); gently finger vortex.
   b. 50 µL of 2.5 *M* $CaCl_2$; gently finger vortex.
   c. 20 µL of 0.1 *M* spermidine; gently finger vortex.
3. Incubate on a continuous vortex mixer for 10 min.
4. Pellet by centrifugation at 13,000*g* for 5 s. Remove and discard the supernatant.
5. Add 140 µL of isopropanol, finger vortex, and centrifuge as previously; then remove and discard the supernatant.
6. Resuspend in 48 µL of isopropanol by gentle pipetting up and down or finger vortexing.
7. Dip the microcentrifuge tubes into an ultrasonic cleaner three times for 1 s each.
8. Finger vortex to homogenate the DNA-coated microcarriers in the suspension and spread 6 µL in a circle approx 1 cm in diameter onto the center of a macrocarrier/holder assembly, which is contained in a Petri plate with desiccant (*see* **Note 16**).

3.3. Bombardment

1. Read the instrument manual and follow the manufacturer's directions and safety precautions. All users should wear safety glasses. A hair net and latex gloves are recommended to reduce the risk of microbial contamination to the plant samples.
2. Set the PDS-1000/He to the following parameters (*see* **Note 17**): 1300 psi (8963.2 kPa) helium (200 psi [1378.9 kPa] above the desired rupture disk value), 1 cm distance between the rupture disk and macrocarrier, 1 cm macrocarrier flight distance, 12 cm of target cell distance, 28-in. Hg (94.8 kPa) vacuum. Sterilize the chamber and all components with 70% ethanol (some components may be autoclaved per the manufacturer's instructions).
3. Place a rupture disk that has been dipped in isopropanol into the retaining cap. Place cap on the end of the gas acceleration tube and tighten.
4. Insert a sterile stopping screen into the support. Load a macrocarrier/holder unit with the microcarriers facing down, on top of the fixed nest. Tighten the macrocarrier cover lid and reposition the microcarrier launch assembly in the bombardment chamber.
5. Place uncovered Petri plate containing target cells into the chamber and close the door.
6. Activate the PDS-1000/He unit by first pressing the vacuum switch. When the pressure reaches 28 in. Hg (94.8 kPa) move the vacuum switch to "hold" (*see*

Note 18). Press the "fire" button until the rupture disk bursts. After bombardment, release the vacuum by moving the switch to "vent." Remove the Petri dish with bombarded cells from the chamber; replace the lid and place in an opaque plastic box. Discard the used rupture disk, macrocarrier, and stopping screen.

3.4. Postbombardment Reduction of Medium Osmotic Potential

1. Incubate all Petri plates (bombarded cells and nonbombarded controls) in the dark at 23 ± 1°C for 2 d to allow cell repair and DNA integration.
2. Approximately 16 h after bombardment, begin to reduce the osmotic potential of the culture medium by transferring the cells and bombardment medium below as a unit (using Sharkskin filter paper with attached tabs) to Petri plates containing 10 mL of 1/2 MS-HF medium without osmotica.
3. At approx 24 h postbombardment, transfer the cells and bombardment medium (using Sharkskin filter paper with attached tabs) to Petri plates containing 20 mL of 1/2 MS-HF medium without osmotica, leaving the 10 mL of medium from the previous transfer behind (discard).

3.5. Analysis of Transient and Long-Term GUS Expression

Transient GUS expression is assayed in a portion of the plates 48 h after bombardment. A plate of negative control cells (nonbombarded or bombarded without the *uidA* gene) should be assayed as well. This assay is destructive (*see* Chapter 14) (*see* **Note 19**). The analysis should be repeated in other plates on a monthly basis for 3–6 mo to evaluate rates of long-term GUS expression as an indication of stable transformation.

1. Using sterile forceps, transfer filter papers with cells to empty Petri plates and place 600 µL of X-gluc solution on top of the cells.
2. Incubate at 37°C overnight. Transformed cells will turn blue.
3. Count the number of blue spots per plate using a stereomicroscope. A black grid on transparent plastic (homemade) placed either above or below the cells aids counting. When transformation rates are high, only a portion of the cells on the plates needs to be counted.

3.6. Embryo Selection, Germination, and Regeneration (see **Note 20**)

1. Two days after bombardment, cells should be transferred to selective medium with 10 mg/L of Km. Using sterile forceps, lift the original Whatman no. 2 filter paper supports with cells from the bombardment medium and place on top of selection medium. Wrap the Petri plates with Parafilm and incubate at 27 ± 0.1°C in the dark for embryo induction. After 4 wk (and every 4 wk thereafter) transfer the cells with supporting filter paper to fresh selective medium with 15 mg/L of Km. Putative Km-resistant embryos should be visible beginning approx 6–8 wk after bombardment.

2. Harvest individual embryos with a 1–2 cm long radicle from Km-selective medium and place directly on embryo germination medium in Petri plates. Wrap the plates with Parafilm and incubate embryos for 4 wk at 23 ± 1°C with low light intensity (10 μE/m^2/s), 14:10-h light/dark (L/D) photoperiod (*see* **Note 21**).
3. Transfer embryos every 4 wk to fresh embryo germination medium in baby food jars. Wrap jars with Venting tape and incubate at 23 ± 1°C with increased light intensity (50 μE/m^2/s), 14:10–h L/D photoperiod.
4. Transfer germinated embryos with elongated roots and open green cotyledons to Magenta boxes containing plant growth medium. Incubate embryos at 23 ± 1°C for root elongation and shoot formation. Transfer to fresh medium every 4 wk.
5. Maintain regenerated plants on plant growth medium in Magenta boxes at 23 ± 1°C for multiplication. Transfer shoots to fresh medium every 6 to 8 wk.

4. Notes

1. Embryogenic cell cultures are often the best tissue to use for biolistic transformation because they can be spread to provide a uniform target of cells, and because they have a high capacity to regenerate into plants. We use proembryogenic cells that are finely divided because they spread easily on the filter papers. Small cell clusters also are effective for selection of transformants as fewer nontransformed escapes result.
2. Supplementing the bombardment medium with osmotica (mannitol/sorbitol) resulted in higher rates of stable transformants for all suspension cultured cells we have tested. However, the benefits of osmotica are less clear when intact tissues such as leaves or whole embryos are used. It is believed that plasmolysis of the cells reduces damage by preventing leakage of protoplasm from bombarded cells ***(17,18)***. Partial drying of cells has also been used ***(19)***.
3. Bio-Rad also sells gold particles in different sizes, with 0.6 μm and 1 μm being most applicable for plant cell transformation. Tungsten particles work well for many plant species and are much less expensive. However, the size is heterogeneous and tungsten may degrade DNA or be toxic to plant cells ***(20)***. See Bio-Rad bulletin US/EG Bulletin 2015 for a discussion of particle types/sizes (available at Website: http://www.bio-rad.com).
4. DNA and tungsten particles may stick to the sides of certain brands of microcentrifuge tubes, resulting in loss of particles. We have not tested all brands, but know that Treff tubes work well.
5. DNA should be very pure (free of RNA or protein) or microprojectiles may clump. We purify DNA by CsCl gradient centrifugation or a plasmid purification kit (Qiagen, Valencia, CA).
6. Spermidine stocks can degrade even when frozen, causing dramatic reductions in transformation efficiency. Fresh stocks should be made monthly.
7. Rupture disks come in a range of bursting pressures from 450 to 2200 psi. The most commonly used for plant tissues are 1100 psi. Rupture disks of higher psi impart higher velocity to the macro- and microcarriers, but also cause more tissue damage. These may be appropriate for more sturdy tissue such as leaves.

8. To dispense 10 mL onto the plates, the medium must be spread by swirling the plates, or by pipetting extra medium and then removing medium until only 10 mL remains.
9. It is important to design bombardment experiments to be performed comfortably by the operator so that the experiment is not rushed or critical details overlooked. In our laboratory, with two people working together it is possible to bombard a maximum of 50–60 plates of suspension cultured cells in 1 d. One person prepares the target cells and adds them to Petri plates with bombardment medium, and the second person prepares the DNA-coated microcarriers and the biolistic device. They then work together to perform the bombardment.
10. The cells in the flasks should be divided into multiple flasks as the population increases. There is no specific formula for dividing the cell culture; rather, the transfer technician should develop an eye as to how dense the population should be to maintain a creamy white or light yellow color and a small cell cluster size.
11. Contamination of the original cell culture can be a source of frustration because whole experiments can be lost after the work of bombardment. At each weekly subculture of the cell suspension, samples of media and cells should be streaked onto Petri plates with bacterial growth medium and/or plant growth medium and incubated both at 25°C and 37°C. Just prior to preparing the cells for bombardment, a sample of the cells and growth medium should be placed on a glass slide with a cover slip and examined with a compound microscope. Use phase-contrast optics if available or move the condenser out of focus to observe cells and possible microbes better. To gain experience in observing microorganisms in culture, researchers should practice looking at plant cell cultures contaminated with various organims as well as those known to be clean.
12. The bore of a 5-mL pipet is too small and cell clumps cause blockage. Attempt to minimize cells lost off of the edge of the filter paper while also achieving a uniform spread across the whole filter paper. It takes some practice to achieve a uniform layer of cells on the filter paper.
13. Macrocarriers and holders may be assembled and autoclaved as a unit. However, we have occasionally experienced shrinkage of macrocarriers after autoclaving, resulting in premature slipping of the macrocarriers from the holders. Thus, we prefer alcohol sterilization. Macrocarriers should be kept free of dirt and oil (from fingers).
14. We follow the protocol suggested by Sawant et al. ***(21)***, in which heating gold particles was shown to reduce particle agglomeration and significantly enhance transformation.
15. Particles settle out of suspension quickly. When removing aliquots, work quickly and vortex-mix often. As stated by Birch and Franks ***(22)***: "The importance of consistent technique in precipitating the DNA onto the microprojectiles and loading the accelerating apparatus should not be underestimated. Two operators of a single apparatus may obtain a 100-fold difference in transformation frequencies because of slight variations in technique at this stage."

16. Finger vortex-mix each time before aliquoting microcarriers. It is important to place macrocarriers in a desiccator to dry immediately after they are loaded. Exposure to high humidity during and after drying may result in clumping of the particles and tight (sometimes irreversible) binding to the macrocarrier ***(23)***. Use DNA-coated macrocarriers within 2 h after preparation.
17. The gene gun settings are critical for success and should be checked before each bombardment. We use a prototype of the Bio-Rad instrument in which the settings are adjustable over a larger range. However, the settings we describe here can be achieved with the Bio-Rad unit. We use a small plastic ruler to measure the distances. Higher particle velocities are obtained with higher helium pressures, and shorter rupture membrane to macrocarrier and macrocarrier to target cell distance. One must be cautious in interpreting transient expression assays because the factors that increase particle velocity also increase the shockwave to the tissue and may actually decrease stable transformation. The settings we use are standard in our laboratory for cell suspension cultures. With intact tissues it may be desirable to increase helium pressure, decrease target cell distance, or bombard each sample multiple times to improve penetration of the particles into the tissues. The reader is referred to several reviews for further discussion on the optimization of biolistic parameters ***(4,5,7,22)***.
18. Leaving the Petri plate at or near 28 in. Hg (94.8 kPa) can allow medium to boil and flip out of the plate. This problem can be avoided by using slightly lower vacuum, by increasing the concentration of gelling agent in the medium, or by letting medium set for 2 wk before use.
19. The green fluorescent protein (*gfp*) gene is another commonly employed reporter gene whose assay by UV light is nondestructive to the cells (refer to Chapter 15).
20. The procedures and growth media we describe here have been used for *V. vinifera* cultivars 'Chardonnay,' 'Merlot,' and 'Pinot Noir' in our laboratory. Other grapevine species and cultivars have not been tested with this protocol. Researchers should use the optimal embryo and plant growth medium for the genotypes they are working with. Similarly, the type and concentration of selective agent needs to be optimized for each genotype and tissue (even for each cell culture line).
21. Embryos could be incubated either at 4°C in the dark for 2 wk for chilling treatment ***(24)*** and then incubated at 23 ± 1°C with low light intensity (10 $\mu E/m^2/s$), 14:10-h light/dark (L/D) photoperiod, for an additional 2 wk, or incubated at 23 ± 1°C with low light intensity for 4 wk. In our laboratory, we did not find statistical differences between the two treatments.

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