US/EG Bulletin 2087

Biolistic® Transfection of Organotypic Brain Slices and Dissociated Cells

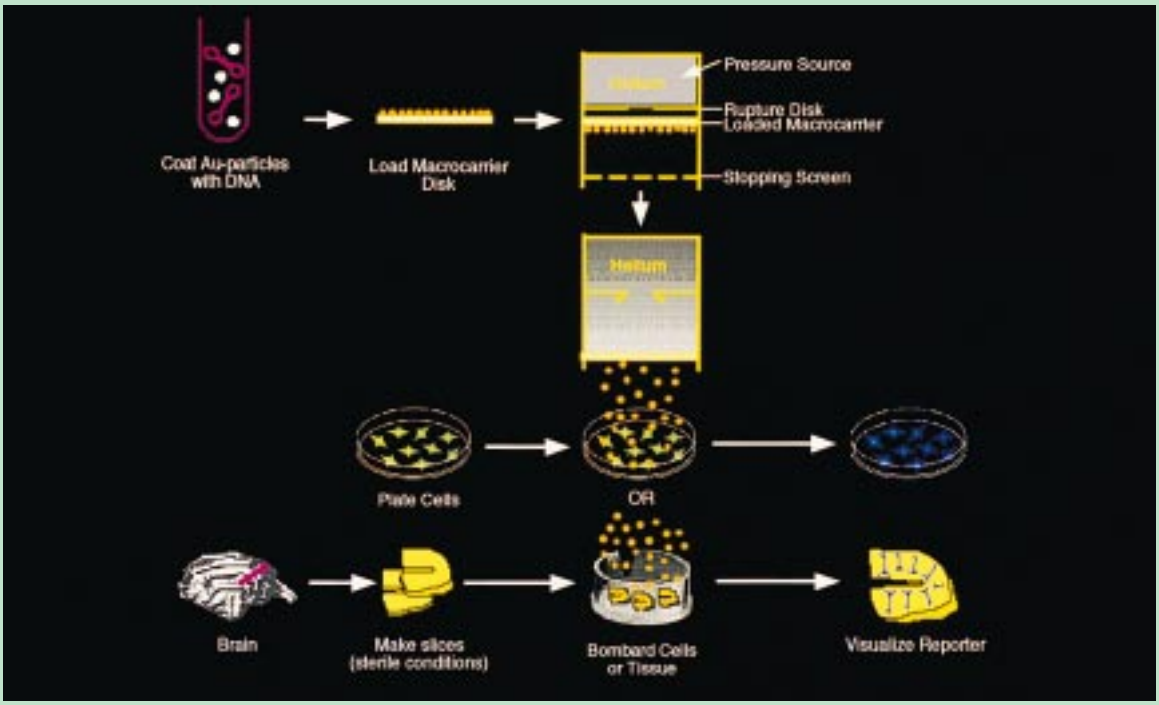

Fig. 1. Schematic of the basic protocol for Biolistic transfection of cell and brain slice cultures.

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Introduction

The introduction of genetic material into cells and tissues of interest remains a rate-limiting step for molecular investigations in many fields, especially neurobiology. Many different methods have been developed to transfect cells—DNA-mediated gene transfer, microinjection, electroporation, lipofection, and viral transfection—and for many cell types, gene transfer using these methods has become a routine tool for studying gene regulation and function. However, many other cell types, particulary postmitotic neurons, have not been easily transfected by these methods.

In the late 1980's, John Sanford, Stephen Johnston, and colleagues developed a device that could potentially allow transfection of such resistant cells (Klein *et al.*, 1987; Johnston, 1990). These inventors called this method "biolistics", an anagram for biological ballistics that describes the process of shooting biological materials into living tissue. Biolistic transformation, which involves accelerating DNA-coated microprojectiles directly into cells, was originally designed to circumvent difficulties in transfecting plant cells. In the eight years since the method was first reported, Biolistic bombardment has also been proven effective in transfecting a wide variety of animal tissues as well as in both eukaryotic and prokaryotic microbes, mitochondria, and microbial and plant chloroplasts (Johnston, 1990; Klein *et al.*, 1992; Pecorino and Lo, 1992; Jiao *et al.*, 1993).

To study the signals which regulate neural development and function, we use both neuronal cell lines (such as PC12 cells) and brain slice cultures. We have applied the Biolistic technology to transfect these preparations since both are difficult to transfect by conventional methods. Here, we provide information on optimizing the Biolistic parameters for transfection of dissociated cells and cultured brain slices (see Bio-Rad instruction manual; Arnold *et al.*, 1994; Lo *et al.*, 1994). Once optimized, Biolistic transformation is a reliable and efficient method for studying gene function in both cell lines and brain slice cultures.

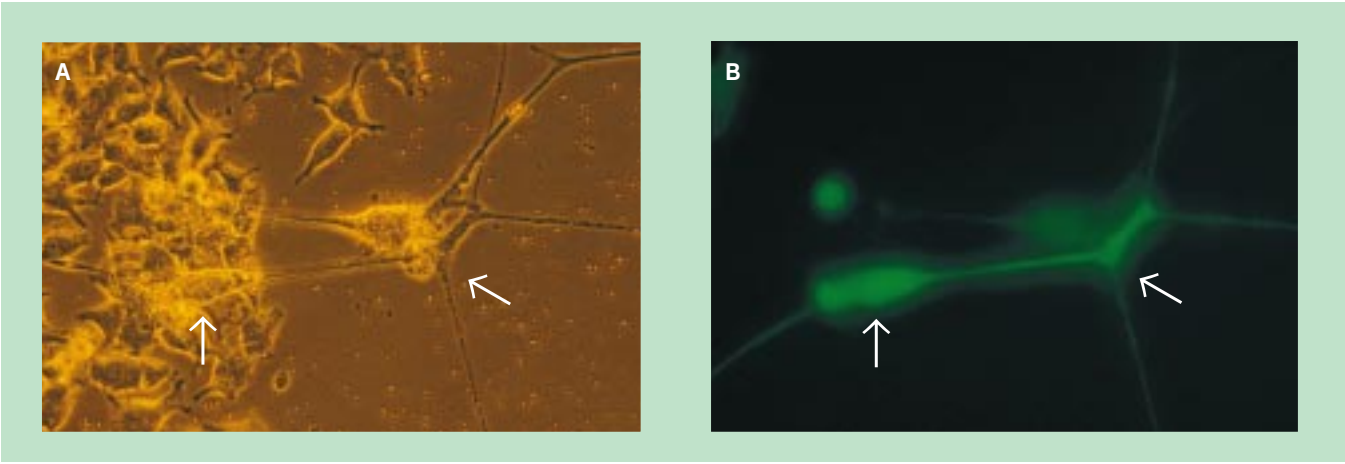

Fig. 2. Dissociated PC12 cells co-transfected with DNA constructs encoding the TrkC receptor. Green-Fluorescent Protein responded to the TrkC ligand NT-3 by extending neuronal processes (A) and were also green under fluorescent excitation (B).

Methods and Results

In general, particle bombardment consists of three steps: coating microprojectiles with DNA, drying them onto a macrocarrier disk and, finally, propelling them into target cells or tissue. The macrocarrier disk is accelerated with high-pressure helium into a stopping screen, which frees the microprojectiles to bombard the tissue. Cells penetrated by a microprojectile are likely to become successfully transfected.

The details of performing these procedures are described in the PDS-1000/He Biolistic instruction manual from Bio-Rad. We have made several modifications to these basic procedures that allow us to apply Biolistic bombardment to transfection of both neuronal cell lines and brain slice cultures (Figure 1).

Transfection of Neuronal Cell Lines

Culture Preparation

In accordance with requirements for each cell line, cells are plated on collagen-coated or untreated tissue culture plastic. To increase the number of successful transfections, the density of the cells should be high. Cells may be transfected as soon as they have adhered securely to the substrate.

Coating Microprojectiles with DNA

1.6 µm gold particles are prepared using the guidelines for coating microprojectiles provided in the Bio-Rad instruction manual, except for two modifications. First, in the agglomeration step, we vortex the gold for a longer period of time to reduce clumping—(4 minutes for each wash instead of 2 minutes as recommended). Second, after all of the reagents are added to the gold in the DNA precipitation step, we vortex for 2 minutes, then intermittently for the next 20 minutes. The additional time in this step allows for improved coating of the gold particles.

Coating Macrocarrier Disks with Gold

Transfection of dissociated cells requires only a few simple modifications to the standard Biolistic bombardment protocol. Because the percentage of successfully transfected cells varies with the density of gold particles as well as with cell density, we typically use 1.5 times the standard microprojectile recipe to coat six macrocarrier disks (*i.e.* 3 mg gold, 5 µg plasmid/macrocarrier). At greater than two or three times the standard density, however, cell death becomes significant. For co-transfections of two different expression constructs, one-half of the standard amount of each DNA construct is used, such that the total amount of DNA used per recipe remains the same.

Transfecting Cell Lines

To transfect dissociated cells, the media is removed from the culture dish as thoroughly as possible without completely drying the cells; aspirating media into a sterile serological pipette works well. Blasting should proceed quickly from this point, to prevent dessication of the cells. The aspirated culture dish is placed approximately 9 cm below the launch assembly. A vacuum of 25"-27" Hg is pulled, and the cells are bombarded at a rupture pressure of either 450 or 900 psi. The higher pressure is more effective at propelling the gold particles into the cells; however, it may also cause slightly more cell death. Once the cells have been transfected, the media is replaced gently into the dish. Some cells may detach from the dish at this point, but the majority of them will reattach to the substrate and remain healthy.

Neuronal Cell Line Results

We routinely use biolistics for transfecting into neuronal cell lines (*e.g.* PC12 cells) various DNA constructs such as those expressing lacZ, Green-Fluorescent Protein (GFP; Chalfie *et al.*, 1994) or the Trk neurotrophin receptors. Additionally, we are able to efficiently co-express combinations of these plasmids, which is useful for confirming successfully transfected cells and normalizing overall transfection efficiency.

Expression of the transfected DNA constructs can be assayed for by their color (such as the marker GFP or the β-galactosidase substrate, X-gal) or by the morphological change they induce in the cells. Following transfection of single or multiple types of DNA constructs, dissociated cells express the appropriate protein products by 24 hours after transfection; expression persists for a minimum of 4 days. In general, the presence of a gold particle in the nucleus of a cell confirms successful transfection. Under optimal conditions, up to 20% peak transfection rates can be achieved.

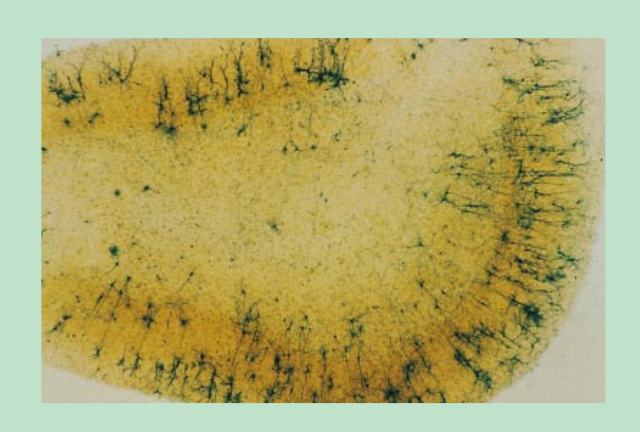

Fig. 3. Low-power view of a P14 slice of ferret visual cortex transfected with a lacZ expression construct and allowed to grow for 36 hours.

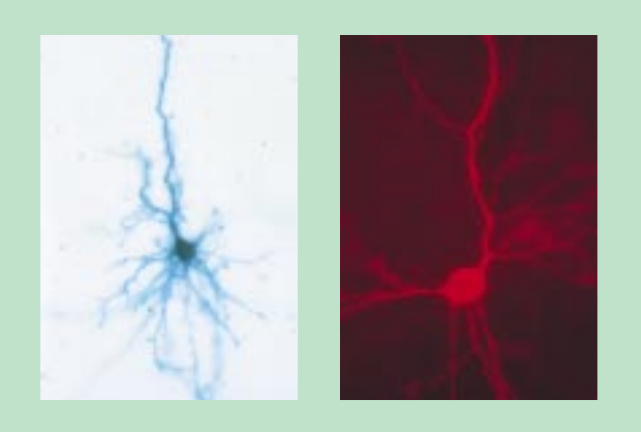

Fig. 4. (A) A layer 5 pyramidal neuron from a slice of P14 ferret visual cortex transfected with a lacZ expression construct and allowed to grow for 36 hours. This neuron was visualized using the substrate for b-galactosidase, X-gal. Note the gold particle in the nucleus (from Lo *et al.*, 1994). (B) A layer 4 pyramidal neuron treated as in (A) but visualized with immunocytochemistry to the b-galactosidase enzyme (from Lo *et al.*, 1994).

A typical result is depicted in Figure 2, where cultured PC12 cells, which normally do not express the neurotrophin receptor TrkC, were co-transfected with two expression constructs. One construct contained the rat cDNA for the TrkC receptor (provided by Regeneron Pharmaceuticals); only the cells which expressed this construct extended processes in response to NT-3 (the neurotrophin ligand for TrkC). The second construct encoded the Green-Fluorescent Protein; cells expressing this construct fluoresced green. In this image of live cells, taken three days after biolistic co-transfection and treatment with NT-3, four cells expressed GFP. Notably, three of the four cells grew extensive processes in response to NT-3 treatment, while those cells which were not green were non-responsive. Such experiments illustrate the high efficiency of co-transfection using Biolistic bombardment.

Transfection of Organotypic Brain Slices

Brain Slice Culture Preparation

Under sterile conditions, 400 µm-thick coronal slices of postnatal day 14 (P14) ferret and P0-P5 rat cortex are prepared as described elsewhere (McAllister, Lo, and Katz, 1995). We use a short-term interface culture system described originally by Stoppini *et al.*, 1991, in which the slices are placed on Millicell® inserts so that the top of the slice is exposed to the incubator atmosphere (5% CO_2 and 36 °C) while the bottom of the slice contacts the culture medium. Although we generally assay these slices after 36 hours, if fed every 3 days with fresh media, they will continue to survive for weeks to months in culture, forming layer-specific connections (L.C.K., unpublished data). As soon as possible after slicing, we bombard the slices, then culture them for 36 hours before visualizing the transfected gene product.

Microprojectile Preparation

The protocol for coating 1.6 µm gold particles and loading macrocarriers is the same as described above for transfecting cell lines, except that for slice transfection, we never use more than one standard recipe of gold for 6 shots. Standard recipe = 50 µl of a 60 mg gold/ 1,000 µl distilled H_2O stock solution.

Transfecting Brain Slices

We have made several modifications to the procedure recommended by Bio-Rad which are crucial to obtaining healthy, transfected neurons in brain slices, although these changes are not important for transfection of cell lines. The primary issue in determining slice health is balancing a sufficiently high helium pressure for gold particle penetration of the slice with the need to minimize the tissue-damaging shock wave resulting from high helium pressures. For slices, we have found 1,100 psi of helium to be optimal. Three modifications have been applied to decrease the damaging effects of the rupture shock wave. First, a 3" square piece of nylon mesh (95 µm mesh opening, 39% open area; Small Parts, Miami Lakes, FL) is cut and attached to the base of the launch assembly, serving to baffle much of the helium shock wave. Second, the Millicell insert containing the slices is placed onto an inch square slab of agarose (1.85%, 0.25" thick) on a cooled aluminum block (approx. 2"x 2"x 0.5") on the target tissue shelf 9 cm away from the launch assembly. The agar acts to absorb the residual shock wave while cooling ameliorates secondary neuronal trauma. Finally, the chamber is flushed with helium through a custom-fitted access valve prior to pulling the final vaccuum so that the residual gas is helium and not air. After the tissue is placed in the chamber, the chamber vacuum is drawn down to 23" Hg. The access valve is then opened and the chamber is refilled with helium from a second tank. Subsequently, the chamber vacuum is reduced to 25" Hg and held until the particles are accelerated into the tissue. Reducing the density of the overlying gas both decreases the drag on the gold particles and lessens the force of the shock wave.

Brain Slice Results

Following Biolistic transfection, neurons and glia expressed β-galactosidase at high levels as detected by X-gal histochemistry (see Lo *et al.*, 1994). Figure 3 shows a low-power view of a slice to illustrate the number of transfected cells in a slice—generally several hundred cells are transfected per slice, located throughout

the entire depth of the cortical plate. Expression can be detected as early as 8 hours post-transfection and increases to fill all of the small processes of both the dendrites and axons of the neurons by 36 hours (Figure 4). Expression lasts for at least 7 days, although the end-point of this transient transfection was not determined. In all transfected cells, a single gold particle can be seen in the nucleus (Figure 4).

Because fine dendritic processes cannot be resolved using X-gal staining (its chromogenic reaction product diffuses some distance away from the enzyme), we visualized these transfected neurons using immunostaining to locate β-galactosidase (Lo *et al.*, 1994; primary antibody obtained from 5 Prime-3 Prime; secondary rhodamine-conjugated goat anti-rabbit antibody from Boehringer-Mannheim). Figure 4 illustrates how effectively immunostaining increases the resolution of the fine processes of the neurons in these slices. Using this method of visualization, even the axons of transfected neurons were visualized for millimeters across the slice, terminating in clearly defined growth cones (Lo *et al.*, 1994).

Discussion

A number of factors affect the success of Biolistic transfection in a given tissue (see Methods and Results above and Sanford *et al.*, 1993 for a more comprehensive review). These parameters must be optimized for each cell and tissue type, and controls must be designed to ensure that bombardment process is not causing cell or tissue damage. For organotypic brain slices, the most important parameter is culture health. If slices are unhealthy for any reason, levels of transfection will be reduced. Once the specific parameters of the Biolistic transfection are optimized for a particular tissue type, the method itself is quite reliable and reproducible. More often than not, if no transfected cells are obtained, it is the health of the cultures that is the problem.

Successful transfection of neuronal cell lines can be achieved with a fairly wide range of Biolistic parameters. The transfection rate of these cells is very reliable, even for co-transfection of multiple plasmids. While transfection rates vary according to the density of cells plated, the amount of gold used in each blast, the efficiency of the expression construct, the exact blasting pressure, the amount of vaccuum pulled, and protecting the cells from the shock of the blast do not seem to be crucial for successful transfection of cell lines.

Summary

We have shown that Biolistic bombardment can be reliably used to transfect neuronal cell lines and postmitotic neurons in intact brain tissue. Once the specific parameters of the Biolistic transfection are determined, this method is efficient, reproducible, and does not require advanced molecular biological facilities for its application. Significantly, Biolistic bombardment allows the time and location of transfection to be determined by the age of the animal from which slices are made and allows gene manipulation of individual cells in otherwise normal background tissue, thus circumventing many of the problems of interpreting data from transgenic and knockout animals.

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Acknowledgements: We would like to thank Stephen Johnston for his continued support and advice in applying Biolistic transfection to cell lines and brain slices. Thanks also to Regeneron Pharmaceuticals for providing neurotrophins, Trk receptor cDNA, and PC12 cells; to Lloyd Green for his gift of PC12 cell lines; and to Martin Chalfie for providing GFP cDNA.

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