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Gene Delivery to Mammalian Cells by Microinjection

Robert King

1. Introduction

Microinjection—that is, the direct-pressure injection of a solution into a cell through a glass capillary—is an effective and reproducible method for introducing exogenous material into cells in culture. The method has been in existence almost as long as there have been microscopes to observe the process. Barber ***(1,2)*** first described the technique that forms the basis for today's microinjection applications. Described here is a simple method for microinjecting individual adherent cells in culture. This application may be used for microinjecting the myriad mammalian cell types that may be encountered in this field.

2. Materials

2.1. Cells

The best cells for microinjecting will be large, easily adherent, with a pronounced nucleus, giving them a tall aspect. For these reasons, PtK1, MDBK, or CHO, all available from ATCC (Manassas, VA), are appropriate for learning good microinjection technique. In theory, any mammalian cell can be injected in the manner described here, although some types provide more challenges than others. Contractile cells such as muscle often change shape rapidly in response to being injected (particularly when calcium is present in the medium), and cells that do not lay flat when cultured may need to be held in place with a second, "holding" micropipet.

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2.2. Micropipet

1. Glass capillary tubing for fabricating micropipet (e.g., Sutter Instrument Company, Novato, CA, Cat. no. BF100-78-10).
2. Micropipette puller for preparing the glass micropipettes (e.g., Flaming-Brown-type puller, Sutter Instrument Company, Cat. no. P-97) (*see* **Note 1**).

2.3. DNA

Any plasmid containing a cytomegalovirus (CMV) promoter-driven reporter gene that may be assayed in individual cells (e.g., green fluorescent protein [GFP] or β-galactosidase) may be used for monitoring the efficiency of microinjection.

1. Prepare plasmid by standard methods and purify using CsCl-ethidium bromide gradient centrifugation ***(3)***.
2. Resuspend the DNA in a buffer-free solution at a concentration of up to 1 mg/mL ***(4)***.

2.4. Microinjection Apparatus

1. Microscope (e.g., Olympus IX-51 with phase contrast optics, 10×, 20×, 40×, objectives, 10× eyepieces, and three-plate moving stage [Olympus America Inc., Melville, NY]) (*see* **Note 2**).
2. Micromanipulator (e.g., XenoWorks micromanipulator, Bio-Rad Laboratories, Hercules, CA, Cat. no. 165-2802) (*see* **Note 3**).
3. Microinjector (e.g., XenoWorks "digital" microinjector, Cat. no. 165-2805 [110/120 volt] or Cat. no. 165-2806 [220/240 volt] [Bio-Rad Laboratories]) (*see* **Note 4**).

3. Methods

3.1. Cell Preparation

Inoculate the cells onto coverslips (*see* **Note 5**) in 35- or 60-mm tissue-culture plates. Use the appropriate growth medium for the cells (e.g., Dulbecco's Modified Eagle's Medium; DMEM) buffered with 10–20 m*M* HEPES, pH 7.5 (*see* **Note 6**). Inoculate the cells at a low density so that there is minimal contact between adjacent cells.

3.2. Micropipet Preparation

Immediately before use, fabricate a number of micropipets from 1-mm outer diameter, thin-walled capillary glass with an inner filament (the filament aids filling the very tip of the micropipet with injection solution) using a micropipet puller according to the manufacturer's directions.

3.3. DNA Preparation

1. Centrifuge the purified DNA solution at 10,000–15,000*g* for 10 min prior to loading the supernatant into the micropipet.
2. Using a 27G needle attached to a 1-mL syringe, load a few microliters of DNA solution through the back-end of the micropipet.
3. Fit the micropipet to the microinjector and lower the micropipet tip into the culture medium. Too long a delay between loading the micropipet and immersing the tip in the medium may cause the liquid in the tip of the micropipet to evaporate, resulting in blockage.

3.4. Microinjection Apparatus Set-Up

1. Ensure that the micromanipulator is set with all three axes at the center of their movement ranges. This ensures that one does not run out of travel during an experiment.
2. Place the dish containing the cell-covered coverslips in the center of the microscope stage, select the lowest power objective available, and focus the microscope on the cells. The plane of focus now corresponds with the bottom of the dish.
3. Rack the microscope objective up a few millimeters so that the plane of focus of the microscope is now a little above the cells.
4. Insert a microinjection pipet into the micropipet holder. Place the holder in the clamp of the micromanipulator and, using the pitch adjustment on the clamp, align the micropipet so that its tip projects into the optical axis of the microscope. Take great care not to touch the micropipet tip against anything—it will break very easily.
5. Look down the microscope while using the micromanipulator joystick to position the micropipet tip in the center of the field of view. Bring the micropipet tip into focus by moving the Z-axis of the micromanipulator, **not** the focus control of the microscope. The micropipet tip should now be in the medium and a few millimeters above the cells. By focusing the microscope up and down, one can view the micropipet tip or the cells.
6. Slowly and very carefully, begin to rotate the Z-axis of the micromanipulator, lowering the micropipet tip toward the cells. Follow the tip down toward the cells with the focus of the microscope.
7. As the cells come into focus, stop lowering the micropipet tip; if the tip touches the coverslip, it can break.
8. Set the microinjector to provide a burst of injection pressure for as long as the footswitch is held down (so-called “continuous-flow” injection) (*see* **Note 7**). Also, adjust the balance pressure so that a slight trickle of solution is always coming from the micropipet tip.

9. Prior to injecting any cells, try a test injection to ensure that the DNA solution is flowing by pressing the microinjector footswitch (*see* **Note 8**).

*3.5. Injection Procedure (*see *Note 9*)

1. Identify a target cell within the field. Microinjection is a concert of three simultaneous actions:
   a. Twisting the Z-axis control of the micromanipulator joystick, lowering the micropipet tip toward the cell;
   b. Movement of the joystick so that the micropipet penetrates the cell (*see* **Fig. 1**) (*see* **Note 10**); and
   c. Depression of the footswitch of the microinjector to inject the DNA into the cell. Ideally, a small quantity of no more than a few percent of the cell's volume will be introduced and will be visible as a tiny shockwave passing through the cytoplasm.
2. Immediately after injection, reverse the direction of movement of the joystick to withdraw the micropipet and release the footswitch.

4. Notes

1. A micropipet puller is required to fabricate consistent microinjection pipets. The Flaming-Brown-type puller (P-97, Sutter Instrument Company) is a

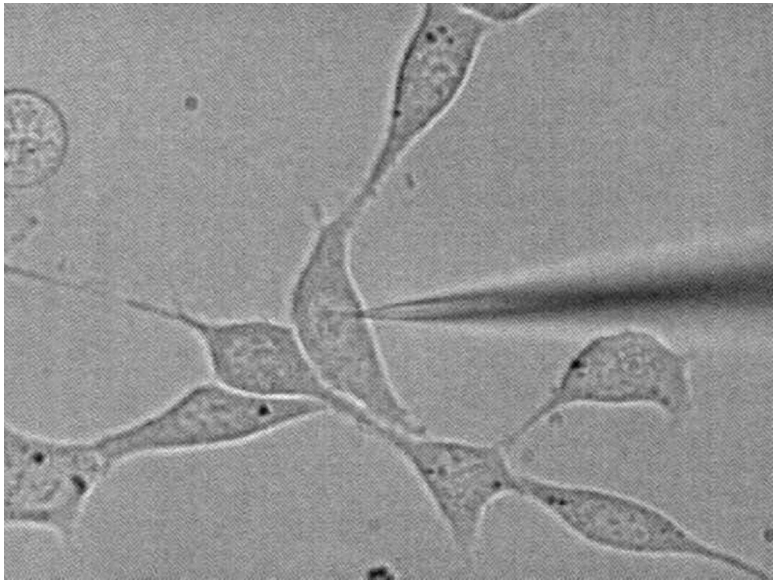

Fig. 1. View through the microscope of a microinjection micropipet entering from the right and projecting down at an approx 45° angle to impale a cultured adherent cell.

sophisticated device that can be calibrated to ensure consistency from one micropipet to the next and produces excellent micropipets for injecting adherent cells. The ideal micropipet will have an internal opening at the tip of less than one micron and have a gentle taper from the tip to the un-pulled section of the capillary.

2. Microscope: In general, an inverted microscope, one whose objective lenses focus beneath a fixed stage, is best suited to microinjection. Although microinjection *can* be performed on an upright microscope, the short working distance between the objective and the specimen make setting the micropipet very difficult. Other good examples of inverted microscopes suited to microinjection are the Nikon TE 2000 (Melville, NY), the Leica DM-IRE2 (Deerfield, IL), and Zeiss Axiovert 200 (Thornwood, NY). For injecting a monolayer of cultured cells, phase-contrast optics produce the best image.
3. Micromanipulator: There are many micromanipulators available for positioning the microinjection pipet, from simple mechanical reduction mechanisms to computer-controlled motorized systems that can interface with the microinjector and microscope. The best micromanipulator will be joystick-operated, comfortable to use over long periods, mechanically stable, and able to move smoothly and responsively.
4. Microinjector: A good microinjector will have the capability to finely adjust the injecting pressure, be able to apply pressure (and therefore inject solution) in discrete pulses or in a continuous stream, and have a high-pressure "clean" function for clearing blockages (which are extremely common) from the tip of the micropipet.
5. Eppendorf Scientific (Westbury, NY) markets "CELLocate," a coverslip with an indexed grid of 175- or 55-micron squares allowing easy identification of injected cells.
6. Carbonate-buffered medium will turn basic upon exposure to the air during microinjection and so should not be used. Most cells will tolerate being injected at room temperature, but if the process is likely to take more than an hour or so the dish should be returned to the incubator periodically. Alternatively, a microscope stage incubator or warm stage insert can be used, both of which are available from the microscope vendor.
7. The precise injection pressure required will depend on a number of factors: the inner diameter of the micropipet tip will determine the flow characteristics and the concentration of the DNA will affect its viscosity, the more viscous the solution, the greater pressure will be required. Trial and error will determine the correct pressure. Start with an injection pressure of about 20–30 psi and slowly increase from there. In general, it is better to work with a higher pressure and spend less time with the microinjection pipet inside the cell (*see* **Note 9**).

8. Under phase-contrast optics the fluid released from the micropipet should be visible as a stream of differing contrast. If no stream is noticeable, try to blow a small fragment of unattached cell around the dish. If no fluid flow can be detected, then the micropipet tip may be blocked. Try cleaning the tip with a quick blast of "clean" pressure and by raising the micropipet tip out of the medium—breaking the surface meniscus can dislodge foreign material.
9. In general, the success of this type of microinjection is highly dependent on operator skill and experience. With practice, quite consistent volumes may be delivered to each cell. The following factors should be borne in mind during the injection:
   a. Spend as short a time as possible with the tip of the micropipet inside the cell, no more than 1 or 2 s.
   b. Enter the cell in a straight line along the axis of the micropipet. Sideways movements will tear the cell membrane and affect the cell's ability to repair the membrane after the injection.
   c. Limit the number of injections performed for each micropipet: cytoplasmic material and fragments of plasma membrane will inevitably adhere to the glass and diminish the performance of the micropipet. After a number of injections, the tip will either block completely or the amount of material on the tip will stick to the contents of the cell and destroy the cell membrane when the tip is withdrawn. Most micropipets may be used for injecting 20–30 cells before there is a diminished performance.
   d. An example of solution that may be used to practice microinjection is 0.01% fluorescein-dextran (Molecular Probes) in calcium-free phosphate-buffered saline (PBS). Successfully injected cells may be identified by fluorescence microscopy ***(5)***.
10. Looking at the dish from the side, the tip of the micropipet should describe a "J" shape with the bottom point of the "J" corresponding to the target being injected, usually the perinuclear area, which is the thickest part of the cell. Under phase contrast, the penetration of the cell can be seen clearly, since a bright white spot appears on the surface as the micropipet tip touches the cell membrane.

Manufacturer's addresses:
Sutter Instrument Company
51 Digital Drive
Novato, CA 94949 USA
(415) 883 0128

Bio-Rad Laboratories, Inc.
2000 Alfred Nobel Drive
Hercules, CA 94547 USA
(800) 876 3425

Olympus America, Inc.
2 Corporate Center Drive
Melville, NY 11747 USA
(800) 645 8160

Molecular Probes, Inc.
29851 Willow Creek Road
Eugene, OR 97402 USA
(541) 465-8300

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