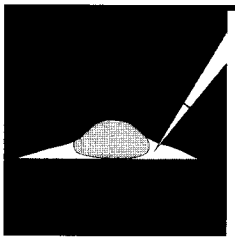

Microinjection of Proteins into Somatic Cells: Needle Microinjection and Scrape Loading

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I. Introduction

Microinjection has been used since the 1920s as a means for delivering foreign molecules into cells. The greatest value of the approach is in allowing direct experimentation on live cells (see also articles by M. Graessmann and A. Graessmann and by Rainer Pepperkok, Rainer Saffrich, and Wilhelm Ansorge). The technique is receiving renewed interest in conjunction with considerable technical improvement and large expansion in the spectrum of probes. In addition, a number of new approaches, such as scrape loading (McNeil, 1989) and electroporation (Glogauer and McCulloch, 1992), have been introduced to complement the direct microinjection approach. To probe different aspects of cells, various types of molecules have been microinjected, including (caged) second messengers, fluorescent probes and indicators, drugs and antibodies, active genes, and antisense nucleic acids. This article focuses on the needle microinjection and scrape loading of protein molecules into cultured somatic cells; however, similar approaches should apply to other types of molecules and cells.

II. Materials and Instrumentation

Collodion bags (Cat. No. 25310 or 25320) and apparatus for vacuum dialysis (Cat. No. 27240) were purchased from Schleicher and Schuell. Centricon filters (Cat. No. 4205 or 4208) were obtained from Amicon. Cells for microinjection were plated in special chamber dishes, with acid-washed No. 2 glass coverslips (Cat. No. 12-543F) as the substrate, as described by McKenna and Wang (1989). In some experiments the medium was covered with a layer of mineral oil (Cat. No. 400-5, Sigma) to minimize evaporation. See McKenna and Wang (1989) for details of culturing cells on the microscope stage.

Microinjection was performed on a Zeiss Axiovert 10 inverted microscope, equipped with a 10× Achrostigmat objective (Cat. No. 44 01 31), a 40× phase-contrast Plan-Neofluar objective (Cat. No. 44 03 51), and 10× eyepieces. Vibration of the table was dampened by installing small tires under the legs. Needles were pulled from Omega-Dot capillary tubing (Cat. No. 30-31-0, Friderick & Haer), using a vertical pipette puller (Model 720, David Kopf). Needles were mounted on a microinstrument collar (Cat. No. 520-145, Leitz) and a micromanipulator (Cat. No. 520-137, Leitz) with a microinstrument holder assembly (Cat. No. 520-142, Leitz). Pressure for microinjection was generated with either an air-filled 10-ml glass

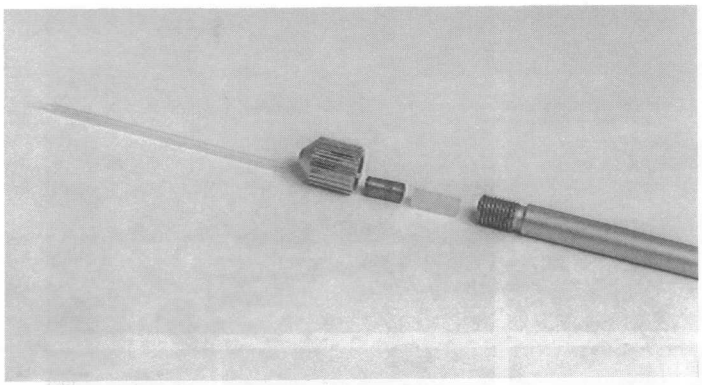

FIGURE 1 Assembly of microneedle and microinstrument collar. The parts from left to right are microneedle, captive nut, brass guide piece, silicon rubber sealing tubing, and metal cylinder. The brass guide piece has one end beveled, which should face toward the sealing tubing. The needle is first passed through the captive nut and the guide piece, and is attached to the silicon rubber tubing. The assembly is then screwed onto the metal cylinder with a gentle tightening pressure.

syringe, coated with a small amount of immersion oil on the inside surface as sealer and lubricant, or with an electronic regulator (custom designed) which maintains a steady air pressure in the range 0.3–1.0 psi. Additional details of the equipment are described in Wang (1992).

A rubber policeman for scrape loading was purchased from Fisher Scientific (Cat. No. 14-105A).

III. Procedures

A. NEEDLE MICROINJECTION

Solutions

1. *Buffer:* Proteins to be microinjected were dialyzed overnight into 2 m*M* Tris–acetate, pH 7.0, or a buffer compatible with both the cell (e.g., low calcium, low buffering capacity, near-neutral pH, limited salt and magnesium, nontoxic) and the protein. Collodion bags were used for the dialysis of small volumes and for the concentration of protein solution by vacuum dialysis. Amicon filters were used for concentration of some proteins. The solution was clarified by centrifugation at 25,000 rpm for 20 min in a Beckman type 42.2 Ti rotor.

2. *Culture medium at 36–37°C.*

Steps

1. Plate cells for 12–48 hr.

2. Scan the dish at a low magnification (e.g., 100×) to choose an area for microinjection. For most experiments, the cell density should be somewhat below confluency to provide adequate space for cells to spread and for maneuvering of microinjection needles.

3. Load protein solution from the back end of the needle with a drawn-out Pasteur pipette. Mount the loaded microneedle on the microinstrument collar (Fig. 1) and the micromanipulator.

4. Apply some pressure to prevent backflow of medium into the needle.

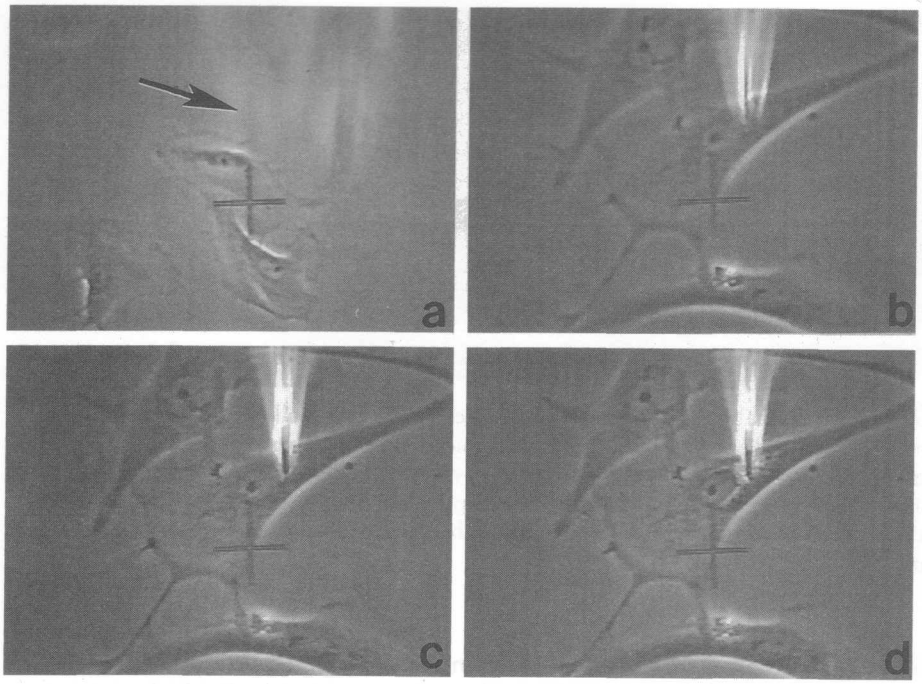

FIGURE 2 Steps in microinjection. (a) Microscope is set at a low power (100×) and is focused on the cell. The needle is at a level higher than the cell and appears as a ghost shadow (arrow). It is carefully brought into the field with a micromanipulator. (b) The magnification is changed to 400×. The needle is positioned above the site of injection and appears slightly out of focus. (c) The tip of the needle is brought into focus and is making a dent on the surface. (d) The delivery of solution starts following a gentle tap at the micromanipulator. The flow induces a local decrease in phase density.

Carefully introduce the needle into the medium. Keep the needle at a level well above cells. The microscope should be focused on cells and the needle should appear as a blurred shadow (Fig. 2a). Bring the needle near the center of the field.

5. Switch to high magnification (e.g., 400×) and focus the microscope on cells. Bring the needle close to focus but still above the level of the cells.

6. Adjust the pressure to about 1/3 psi. The flow of solution can be checked by carefully bringing the needle close to focus and switching to fluorescence optics (if the solution contains a fluorescent marker). Raise the needle back to a higher level.

7. Position a cell to be microinjected directly under the needle (Fig. 2b). Carefully bring the needle to focus, i.e., lower down the needle into the cell (Fig. 2c). Microinjection often starts spontaneously. Otherwise, a gentle tap at the micromanipulator should cause the needle to puncture the cell membrane.

8. The delivery of solution manifests as a change in phase density that spreads out from the needle (Fig. 2d). The volume of injection is controlled by the pressure and by the length of time the needle stays inside the cell. Raise the needle out of the cell to stop the microinjection. Adjust the injection pressure if necessary between cells. An alternative approach is to raise the pressure momentarily after puncture of the membrane, under the control of a timing device (e.g., pressure regulator from Eppendorf).

9. Replace the medium following each hour of injection and at the end of microinjection to correct for any evaporation that may have occurred and to remove materials that spilled into the medium. Alternatively, evaporation can be minimized with an overlay of mineral oil.

Solutions

1. *Phosphate-buffered saline (PBS):* Contains 137 m*M* NaCl, 2.68 m*M* KCl, 8 m*M* Na_2HPO_4, and 1.47 m*M* KH_2PO_4.

2. *Protein solution in PBS:* The concentration is usually several times higher than that targeted for delivery. The optimal concentration should be determined empirically.

3. *Culture medium at 36–37°C.*

Steps

All steps are performed in a sterile hood.

1. Start with subconfluent cell culture on 35- or 60-mm culture dishes. Cells should adhere well to the dish; otherwise, a shorter plating time may improve the viability of the cells after loading (McNeil, 1989).

2. Rinse the dish three times with PBS. Remove PBS thoroughly by aspiration.

3. Add the protein solution to the dish. The volume should be large enough to distribute over the entire surface after tilting the dish. Two hundred microliters is sufficient for 60-mm dishes.

4. Scrape the entire surface of the dish gently with a sterile rubber policeman. This should be finished within 10–20 sec.

5. Add warm medium to the culture dish and transfer cells into a 15-ml conical centrifuge tube (e.g., Falcon Cat. No. 2099).

6. Spin down cells in a benchtop centrifuge.

7. Remove supernatant and resuspend cells in warm culture medium. Plate cells on fresh culture dishes and culture in an incubator for several hours.

IV. Comments

The presence of a fluorescent marker in the injection solution can greatly facilitate microinjection. It allows examination of flow and easy identification of injected cells. Fluorescent dextran (Sigma or Molecular Probes) at 1–5 mg/ml can be used as an inert marker.

It is critical to obtain an optimal shape of the microneedle by adjusting the temperature and force of the pipette puller. Blunt needles are fragile and cause excessive cell damage; a slender taper traps air bubbles during backloading.

Each needle can be used for more than 50 cells, assuming that the solution for microinjection is reasonably clean. The needle will eventually become clogged by materials inside and from contact with the cytoplasm. High-pressure (e.g., 20 psi) pulses can be used to push out small aggregates; however, it is very difficult to push out any air bubbles due to surface tension. Sometimes it is possible to carefully break the tip, by gently touching the glass, and use at a reduced pressure.

The volume delivered by needle microinjection is usually in the range 1 to 10% cell volume. With some practice the variability can be maintained to within a factor of 2. A number of factors can affect the volume, including the pressure, tip size, accumulation of proteins at the tip, and presence of any air bubbles in the needle.

Without proper landmarks, it can be very difficult to find injected cells once the dish is moved away from the site of injection. Cells can be located by marking the

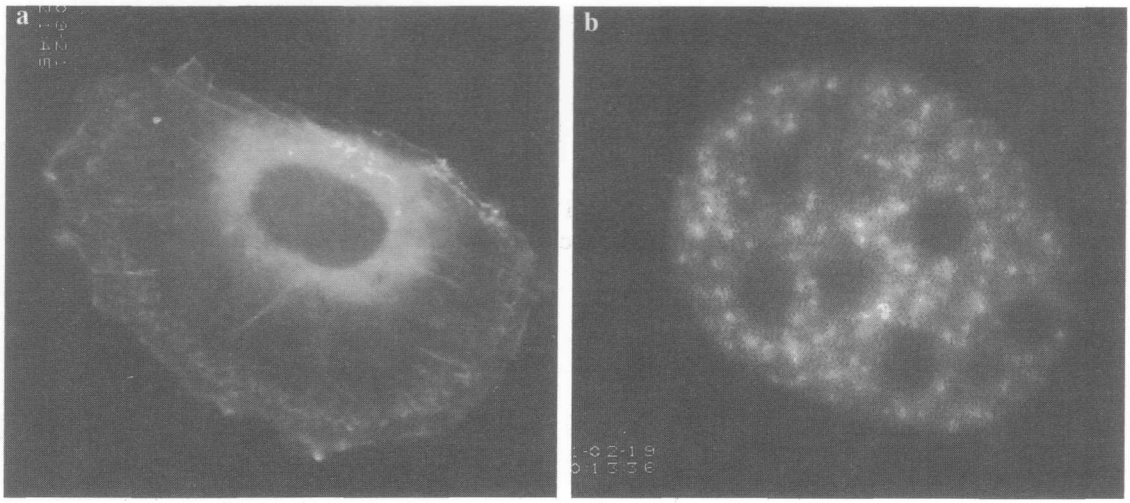

FIGURE 3 Fluorescence images of NRK cells with rhodamine-labeled actin microinjected into the cytoplasm (a) and with rhodamine-labeled human globin pre-messenger RNA microinjected into the nucleus (b). In (a), fluorescent actin becomes incorporated into cytoplasmic structures, with the nucleus appearing as a dark area. In (b), fluorescent pre-mRNA is localized almost exclusively inside the nucleus at numerous discrete sites. Nucleoli appear as dark patches.

coverslip with a diamond pencil and injecting near the mark or by marking the site of injection with a "marker objective."

In some experiments, it may be necessary to recover microinjected cells (e.g., for electrophoresis, polymerase chain reaction). One way to do this is to plate cells on small pieces of coverslips placed inside a culture dish, inject all cells on pieces of coverslips and then remove the pieces with forceps.

Intranuclear microinjections can be achieved in a similar fashion. The needle is first lowered onto the surface above the nucleus, forming a dent on the top cell surface. Injection is started with a gentle tap at the micromanipulator, which causes the needle to penetrate both the plasma membrane and the nuclear envelope. With practice nuclear injections can be achieved with minimal spillage into the cytoplasm. Figure 3 shows an example of cells injected in the nucleus with fluorescent molecules.

V. Pitfalls

Needle Microinjection

1. For well-spread cells, needle microinjection is possible only in the perinuclear phase-dense region. For poorly adherent cells, a second pipette is necessary to hold the cell by suction during microinjection.

2. Physical damage to cells, as during microinjection, induces calcium transients and possibly downstream processes regulated by calcium. Therefore, whenever possible, injected cells should be allowed 1–2 hr for recovery. Controls should be performed to rule out artifacts caused by microinjection.

3. A slight retraction of injected cells is common 5–10 min after microinjection; however the effect should reverse within 30 min.

Scrape Loading

4. Cells must be allowed to recover and respread before microscope observation.

5. Compared with needle microinjection, the amount of delivery varies more extensively among scrape-loaded cells. The yield of injected cells and the extent of cell damage also vary among cell types. Cell damage often manifests as the appearance of large vacuoles.

6. The efficiency of scrape loading varies as a function of molecular weight (McNeil *et al.*, 1984).

7. Some molecules may not be compatible with the balanced salt solution required for scrape loading.

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