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SHORT PROTOCOL No. 47

Step-by-Step Guide: Microinjection of Adherent Cells with the Eppendorf InjectMan® 4 and FemtoJet® 4

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Abstract

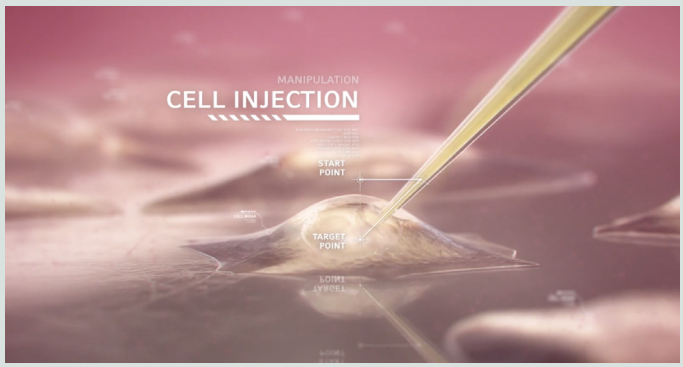
Adherent cell microinjection is a precisely targeted method allowing the delivery of exogenous material like DNA, RNA, or proteins into a living cell by means of a fine glass microcapillary under microscopic control. This technique has proven to be a powerful approach for analyzing the biological activity of specific molecules (e.g., enzymes, peptide inhibitors, neutralizing antibodies) in a single cell.

This step-by-step guide describes in detail the procedure of microinjection into adherent cells using the Eppendorf InjectMan 4 with the FemtoJet 4i. This method optimizes injection parameters ensuring high throughput, a high cell survival rate, and highly reproducible and repeatable microinjections to improve the specificity and reproducibility of your downstream analysis.

Introduction

Microinjection is the transfer of a dissolved or suspended substance into a living cell under microscopic control by means of direct pressure. The method, developed independently by Grassmann [1] and Diacumakos [2], allows the use of single cells as objects to study complex cellular processes, activities, and structures *in vivo*. It facilitates functional analysis of structural proteins, transport processes, protein biosynthesis, enzyme systems, hormonal control mechanisms, signal transduction and cell cycle studies even in difficult-to-transfect cells. Unlike other methods for intracellular delivery, microinjection facilitates the study of fast responding and transient cellular events because injected cells can be identified and observed immediately after microinjection. Applications for microinjection experiments are found in cytology, physiology, molecular biology, neurobiology, and pharmacology.

The technique is based on a simple mechanical process in which a fine glass capillary loaded with the injection sample and driven by a micromanipulator punctures the cell membrane and penetrates into the nuclear or cytoplasmic compartment. After the capillary tip entered into the desired compartment, a controlled and reproducible volume solution is injected into the cell by pressure exerted on the microcapillary via a microinjector. When the injection is complete the capillary is withdrawn from the cell and the membrane reseals with minimal damage to the cell.



Microinjection into adherent cells - a targeted approach to single cell analysis

Typical injection solutions include purified DNA, RNA, antibodies, peptides, oligonucleotides, and endonuclease editing components. It is also possible to transfer nanoparticles or viruses into cells. To visualize and evaluate the success of a microinjection experiment, the molecules may be labeled with fluorescent markers or mixed with fluorescent dyes.

Particles in the injection solution can block the fine opening of the microinjection capillary. Therefore, when they become clogged during a microinjection experiment the capillaries need to be exchanged. The frequency of capillary exchange depends on the nature and concentration of the microinjection sample and the quality of the sample preparation.
Most adherent cells are very sensitive to changes in the pH and temperature of the culture medium. Therefore, the microinjection experiment should be done as quickly as possible so the cells can be returned to the CO_2 incubator before they become stressed.
Eppendorf's semi-automated microinjection system with the InjectMan 4 and the FemtoJet 4i facilitates the microinjection process in several ways.

> Compared to standard microinjection this system enables inexperienced users to quickly become proficient at microinjecting cells.
> The semi-automated axial injection feature allows cells to be rapidly (~20 cells/min) injected with minimal damage to the cell membrane. Because cells can be injected and returned to the CO_2 incubator quickly, it minimizes cell stress from temperature and pH changes. Thus, survival rates are increased.
> The synchronization between the InjectMan 4 and the FemtoJet 4i and the precision of the FemtoJet 4i injections allow for injections which are not only uniform from cell to cell, but also from operator to operator and from one lab to another.

This guide describes microinjecting into adherent cells using the Eppendorf InjectMan 4 with the FemtoJet 4i step by step. It lays out how to set up and use this system to ensure fast, efficient, and highly reproducible microinjections with minimal damage to the cells. By following these steps, users can quickly become proficient at this technique with no previous microinjection experience.

Protocol

I. Preparation of the Sample to be Injected

Sample preparation and handling are crucial for successful microinjection and must be done with great care. Special attention must be given to reduce or remove any particles from the microinjection sample which could block the microinjection capillary.

DNA purification [3]**:** High expression level of microinjected plasmid DNA can be achieved by purification using CsCl ethidium bromide gradient centrifugation [4]. Alternatively, DNA purification kits from different companies can be used. Plasmid DNA concentration for microinjection should be between 20-200 ng/µL depending on promoter activity [5]. Store at -20 °C in small aliquots of 5-10 µL. Gently thaw an aliquot just before the experiment. Centrifuge the sample for 15 minutes at 4 °C (approx. 16,000 x g) and load supernatant directly into the microinjection capillary. Repeated centrifugation of the aliquot is not necessary within an hour if kept on ice.

RNA purification: Any standard protocol is suitable for purifying RNA solutions [4]. Concentrations of 1-2 mg/mL should be used for mRNA and up to 10 mg/mL for total RNA. Store at -80 °C in small aliquots of 5-10 µL. For longer periods it is advisable to dissolve and store the cleaned RNA in alcohol instead of water. Gently thaw each aliquot immediately before the experiment. Centrifuge the sample for 15 minutes at 4° C (approx. 16,000 x g) and load supernatant directly into capillary. Only centrifuge and use each aliquot once.

Protein and antibody purification [6]**:** The purification method which results in the highest activity of the specific protein or antibody should be used. For peptide antibodies raised in rabbits, affinity purification is recommended. Typical concentrations for proteins and antibodies are 1-5 mg/mL. The concentration of the material injected should be 10 to 20 times higher than that required for the optimum in vitro activity because the sample is diluted 10 to 20 times when injected into a cell.
Purified antibodies must be stored in the concentration in which they arise. Shock freeze small aliquots of 5-10 µL in liquid nitrogen then store at -20°C or even better at -80 °C. Azide should not be used. Refrain from repeated freezing and thawing as antibodies lose activity and lead to blockages of the injection capillaries due to agglutination. Thawing should be performed as quickly and gently as possible. Before loading the capillary centrifuge material for 15 minutes at 4 °C (approx. 16,000 x g). Chill supernatant or load directly into capillary.

Oligonucleotide purification: The purification of oligonucleotide solutions is crucial. Cleaning with gel or HPLC is recommended. A concentration of 1-2 mg/mL should be used for injection of antisense oligonucleotides with 10-20 bases. Note that injected oligonucleotides accumulate easily in the nucleus.

Dyes: Fluorescent injection markers [5] for identification and time-lapse imaging of injected cells include: fluorescently labelled dextran, antibodies or bovine serum albumin. Fluorescently conjugated dextrans of 70 kDa and larger can be detected in living cells up to three days after microinjection. Dextran co-injection markers are used in concentrations of 0.5-3.0 mg/mL.
Store at -80 °C in small aliquots of 5-10 µL. Many solutions are sensitive to light thus exposure to direct light should be avoided. Prepare stock solutions at a concentration of at least 10 mg/mL and filter through a 0.2 µm syringe filter.

Microinjection buffers
The buffer which is closest to the physiological milieu of the cell contains:
> 48 mM K_2HPO_4
> 4.5 mM KH_2PO_4
> 14 mM NaH_2PO_4, pH 7.2

Whenever possible this buffer should be used for injection. However, several other buffers have been used without any obvious effect on cell function:

Protein
> Phosphate buffered saline (PBS)
> 50 mM HEPES (pH 7.2), 100 mM KCl
> 5 mM $NaPO_4$ (pH 7.3)

Antibody
> 10 mM NaH_2PO_4, 70 mM KCl (pH 7.2)
> 5 mM $NaPO_4$, 50-100 mM KCl (pH 7.2)
> Phosphate buffered saline (PBS)

DNA, RNA, oligonucleotides
> ddH_2O

Note: To prevent blocking of the injection capillaries all buffer solutions should be filtered through 0.2 µm pore size filters before adding the injection sample.

Before injection experiment
A. Thaw sample (if frozen) and if there is enough, filter through a 0.2 µm filter into a clean microcentrifuge tube.
B. To avoid clogging of the microcapillary by precipitates:
> Centrifuge sample for 20 minutes at approx. 16,000 x g just before loading the microcapillary
> Pipette 10-20 µL of the supernatant into a clean 0.5-1.5 µL tube and close the cap to have the sample ready for loading the microcapillary.

II. Preparation of the Adherent Cell Culture

Cells should be cultured according to the appropriate conditions for the cell line used and should be plated one day before injection to ensure proper cell adhesion. For better cell adhesion TC-treated or biologic coated cell culture dishes can be used. Standard 35 mm and 60 mm petri dishes are commonly used. Low wall dishes offer an improved access of the microinjection capillary. For experiments requiring sensitive fluorescence analysis use a cell culture vessel with low auto-fluorescence, such as a glass bottom cell culture dish, a chamber slide, or a cell imaging coverslip. Cells can also be grown on a coverslip in a plastic cell culture dish, then after microinjection the coverslip can be moved to a coverslip chamber for imaging.
The microinjection process is most efficient when the cells are fully adherent, monolayer, and between 60-70% confluent. Higher densities can lead to overgrowth and morphological changes to the cells making individual cells harder to distinguish and target for microinjection.

Cell handling for microinjection
A. Immediately before microinjection replace the CO_2-buffered cell culture medium with prewarmed CO_2-independent (e.g. HEPES-buffered) injection medium.
B. Fill the dish with a larger volume of injection medium than is typically used for cell culture (see in Figure 1, green arrow). The liquid meniscus formed where the capillary enters the medium refracts light thus degrading image quality. Raising the level of media in the dish greatly improves image quality by moving this area of refraction away from the light path.
C. Immediately after microinjection replace the injection medium with CO_2-buffered medium and place the dish back into the CO_2 incubator.
Note: The total length of time cells spend at room temperature should be as short as possibly, typically less than 30 minutes!

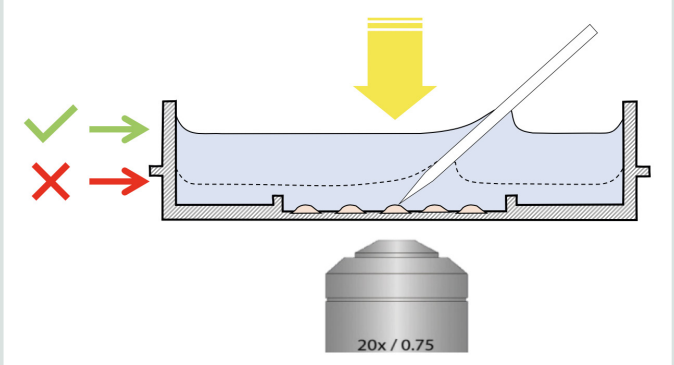


Figure 1: Cell culture dish filled to a higher level with injection medium to reduce image refraction in the light path.

III. Preparation of the Microinjection Workstation

The microscope should be an inverted microscope equipped with phase contrast optics and long working distance 10x, 20x, and 40x dry objectives. The Eppendorf InjectMan 4 micromanipulator is mounted on microscope and is connected to the FemtoJet 4i via an interface cable and the injection tube. The entire workstation should be isolated from building vibrations with an antivibration system to ensure better cell survival and improved microinjection results. The InjectMan 4 described in this guide is mounted on the right side of the microscope. If your system is mounted on the left, simply reverse left and right in these instructions.

A. Before turning on the FemtoJet 4i disconnect the injection tube from the front of the FemtoJet 4i. Then switch on the power. The internal compressor will engage and will build up enough pressure to be ready to use in less than two minutes. When the working display (see Figure 2) appears on the FemtoJet 4i this indicates that the FemtoJet 4i is ready and there is sufficient pressure to inject.

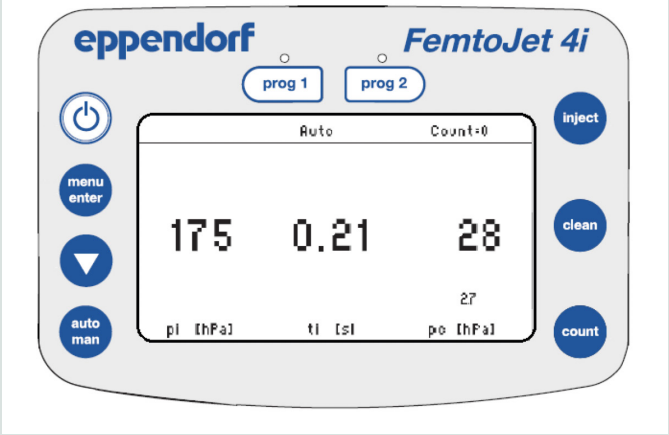


Figure 2: FemtoJet 4i working display showing that the FemtoJet 4i is ready to inject.

B. Adjust the FemtoJet 4i injection parameters. The compensation pressure (pc) remains active between injections and is needed to prevent media from being drawn into the capillary by capillary action. Injection pressure (pi) is the pressure used to expel the sample into the cell. Injection time (ti) is the length of the injection pulse in seconds. Here are some typical starting injection parameters. These parameters must be optimized for the specific cell lines and samples being injected as well as the size of the capillary used in each experiment.

> pi (injection pressure) = 80-120 hPa
> ti (injection time) = 0.2-0.4 sec
> pc (compensation pressure) = 30 hPa

Pressure settings are shown here in hPa (1 hPa = 0.0145 PSI).

C. Switch on the InjectMan 4 and make sure that the "Adherent Cell injection" application is selected. If the FemtoJet 4i is properly connected and ready a note "Injector ready" will appear on the InjectMan 4 display. Press the "home" key on the InjectMan 4 keypad to move the motors to the maximum up and out position. Press the "Back manual" softkey to clear the HOME function in preparation for mounting a new capillary (see Figure 3).

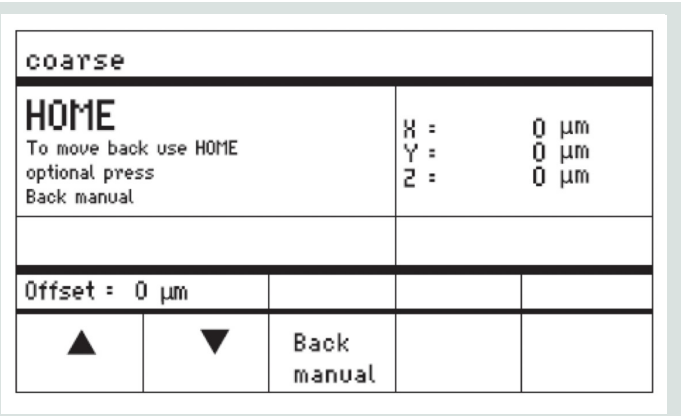


Figure 3: Display of the InjectMan 4 when the HOME function is active showing the "Back manual" softkey.

D. Switch on the microscope and make sure that the transmitted light source is selected and check to make sure that light is shining from the condenser into the light path of the microscope.

IV. Preparation of the Microinjection Capillary

Microinjection capillaries are typically made from 1 mm outer diameter (O.D.) borosilicate glass. One end of the capillary is drawn to a fine tip with an opening size or inner diameter (I.D.) of between 0.2-0.7 µm. Femtotips® are pre-pulled, sterile microinjection capillaries with a uniform opening size of 0.5 µm. They have a screw base making them quick and easy to replace, and they have an inner filling fiber making them easy to back-fill. Microloader™ are special pipette tips designed to allow a small volume of microinjection sample to be back-loaded quickly and easily into Femtotips or other microinjection capillaries.

A. Slowly draw approx. 3.0 µL of the injection sample (supernatant) into a Microloader using an Eppendorf 0.5-10.0 µL adjustable pipette.

B. Insert the tip of the Microloader into the blunt end of the Femtotips or microinjection capillary and slide it as far forward into the capillary as possible being careful not to allow the Microloader to bend enough to kink the plastic (see Figure 4).

C. Slowly pipette the sample into the capillary being careful not to pipette any air bubbles into the column of injection solution. Air bubbles in the column of liquid in the capillary expand and contract when subjected to pressure changes making it difficult to obtain consistent microinjections.

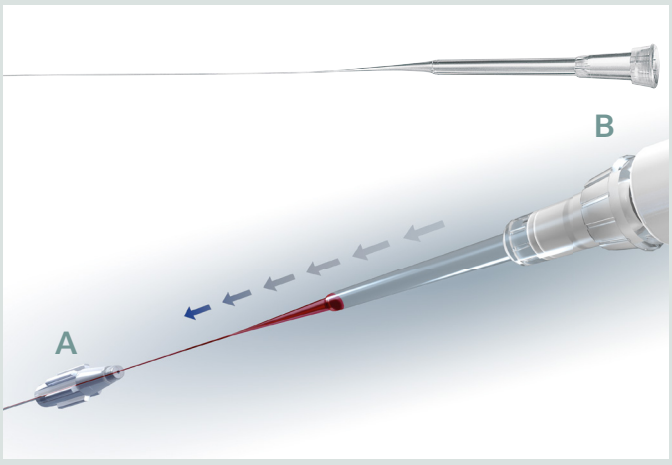


Figure 4: Back-filling of the Femtotips® (A) using the Microloader™ (B).

D. Screw the Femtotips capillary snugly into the Femtotips adapter and screw it onto the front of the Capillary holder 4. Other type of microinjection capillaries can be mounted onto the Capillary holder 4 using the Grip head 4 (see Figure 5).

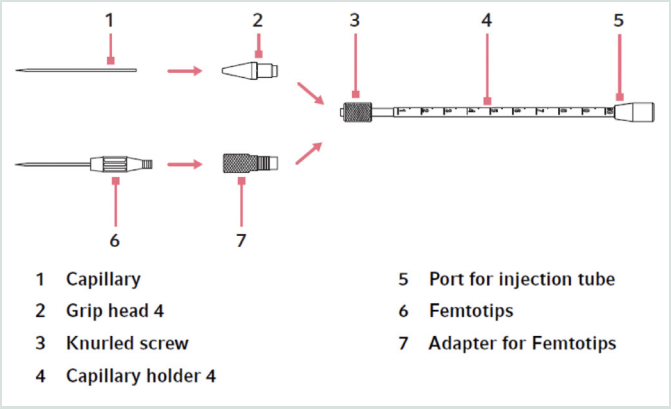


Figure 5: Assembly of the Grip head 4 and Femtotip® adapter onto the Capillary holder 4.

E. Mount the Capillary holder 4 onto the InjectMan 4 micromanipulator by loosening the blue locking knob on the front of the InjectMan 4 and snapping the Capillary holder 4 into place. Tighten the locking knob to secure it in place (See Figure 6A).

F. Set the angle on the angle guide to 45° (see Figure 6B).

G. If needed, loosen the locking knob and slide the capillary holder forward or backward so that the tip of the microinjection capillary is within 2.0 cm of the surface of the cell culture vessel in both the X and Z axes. When done adjusting the position, securely tighten the locking knob.

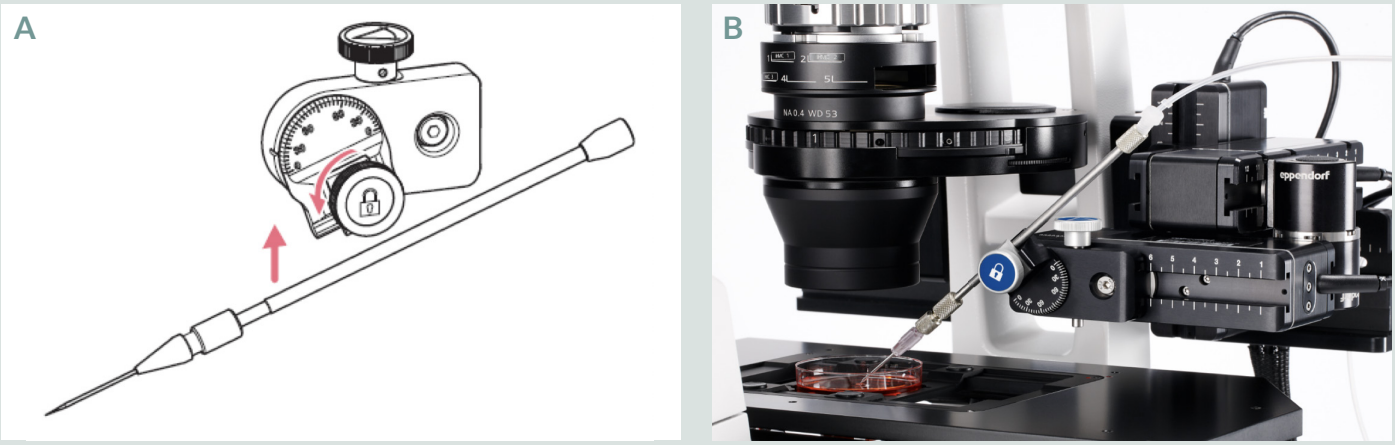


Figure 6: A) Loosen the locking knob to attach the Capillary holder 4 onto the InjectMan 4 micromanipulator.
B) Capillary holder with Femtotips capillary mounted at a 45° angle on the X-motor of the InjectMan 4.

H. With the loaded microinjection capillary in place, press the "menu/enter" key on the front of the FemtoJet 4i twice to close the injection port. Connect the injection tube to the front of the FemtoJet 4i. Check to make sure that the injection tube is tightly connected to the back end of the Capillary holder 4 and press the "menu/enter" key once more to open the port. The compensation pressure is now active and the microinjection system is ready to inject.

J. Being careful not to accidentally break the microinjection capillary, place a dish of cells onto the microscope stage and remove the lid or cover. Move the stage to bring the cells into the field of view of the microscope. Adjust light levels as needed and make sure that you are familiar with the correct condenser setting for each objective.

V. Microinjection: Adjusting the Microscope Focus and Finding the Microinjection Capillary

A. Using a low power objective (5x or 10x), focus the microscope on the cells near the center of the cell culture dish, then focus up approximately 2 mm above the cells.

B. Switch the InjectMan 4 to "coarse" speed and while looking directly at the microscope stage (not through the eyepieces), use the joystick to position the tip of the microinjection capillary into the light path of the microscope above the cell culture dish.

C. Still looking directly at the microscope use the joystick to move the capillary approximately 1 mm above the surface of the media and approximately 1 mm past the center of the field of illumination.

D. Looking through the microscope eyepieces locate the capillary by moving the InjectMan 4 joystick forward and backward in the Y-axis. Watch for the shadow or out-of-focus image of the capillary to come into view. When you see this shadow, use the joystick to center it in the Y-axis of your field of view.

E. If the tip of the capillary is out of view to the left of the field of view use the joystick to move the capillary slowly to the right. Re-center the shadow and refocus the microscope as needed to bring the tip of the capillary into the center of the field of view. Notice that when you focus the microscope on the tip of the capillary the rest of the capillary is out of focus due to the 45°mounting angle.

F. Focus the microscope down part of the way towards the cells so that the tip of the Femtotip is still visible, but out of focus.

G. Turn the joystick head (Z-axis control) clockwise to slowly lower the tip of the capillary down towards the focal plane of the microscope. When the image of the Femtotips is still slightly fuzzy, release the joystick to stop the downward movement and refocus the microscope further down towards the cells. Repeat these steps until the Femtotips capillary touches the surface of the media in the dish.

H. When the capillary touches the surface of the media you will typically see a bright refractive spot. Switch the speed control of the joystick to "fine" and repeat steps F and G until the cells are in sharp focus and the tip of the Femtotip is visible, but still slightly above the focal plane (fuzzy).

Note: It is important on the final step down that you do not bring the tip of the microcapillary completely into focus. It must remain above the surface of the cell culture dish or you risk breaking it.

I. Switch to a higher magnification (20-40x objective) phase contrast dry objective. Focus the microscope on the tip of the Femtotips capillary. While watching the tip briefly press the "clean" key on the FemtoJet 4i. If the tip is unobstructed you should see a strong outflow of liquid.

Note: After several tries if no outflow is seen and if the capillary physically moves each time you press "clean", this is a strong indication that the capillary is blocked and needs to be replaced. To exchange the microcapillary lift the tip approximately 500 µm up by twisting the joystick head counterclockwise. Press the "home" key to drive the microcapillary out of the dish and over its rim, swing out the X-motor to the front, unfix the blocked Femtotips capillary but not the Capillary holder and replace it by a new filled Femtotips capillary. Swing back the X-motor and press again the "home" key to drive the Femtotips capillary automatically back into the microscopic view.

VI. Microinjection: Setting the Z-limit on the InjectMan 4

To use the semi-automated injection function of the FemtoJet 4i – InjectMan 4 system you must first define the correct injection height for your cells. This is referred to as the Z-limit. The Z-limit also acts as a downward movement limit to prevent accidental breakage of the capillary on the surface of the culture dish. A new Z-limit must be set for each culture dish and it may need to be adjusted or reset as you move around the dish which may have a slightly uneven dish bottom. For this protocol we will inject the nucleus of a cell.

A. Focus the microscope on a typical looking cell, which is similar in size and shape and is in the same focal plane as the neighboring cells. It is usually best to focus the microscope on the edge of the nuclear membrane since this gives you a good view of both the nuclear and cytoplasmic compartments.

Note: From this point forward do not refocus the microscope because your focal plane will help guide you to cells which can be microinjected with the same Z-limit.

B. The tip of the capillary should still be slightly above your focal plane. Position the tip of the microcapillary above the nucleus of the cell to be injected.

C. Carefully twist the joystick head clockwise to slowly lower the tip of the capillary towards the cell (see Figure 7.1.). It is often easier to control this Z-movement if you twist and release the joystick in small increments. Continue to lower the capillary until the tip of the capillary just touches the top of the cell membrane. You can see when the tip gets in contact with the cell membrane because the tip will cause a small deformation of the cell surface (see Figure 7.2.) resulting in a phase-bright dot or white halo around the tip of the capillary. If the capillary starts to move sideways as you lower it, it is deflecting on the surface of the culture dish and moved too low – raise it and try again.

D. Once you successfully touch the surface of the cell membrane press the "Z-axis Limit" key on the InjectMan 4 control board to save this height as the microinjection Z-limit. The Z-limit does not contain any X or Y coordinate information, instead it is an artificially defined lower movement plane for the micromanipulator set at the height of your cells. Once you have set a Z-limit, your focal plane will tell you which cells can be injected with this Z-limit, so you should not refocus the microscope until you need to set a new Z-limit.

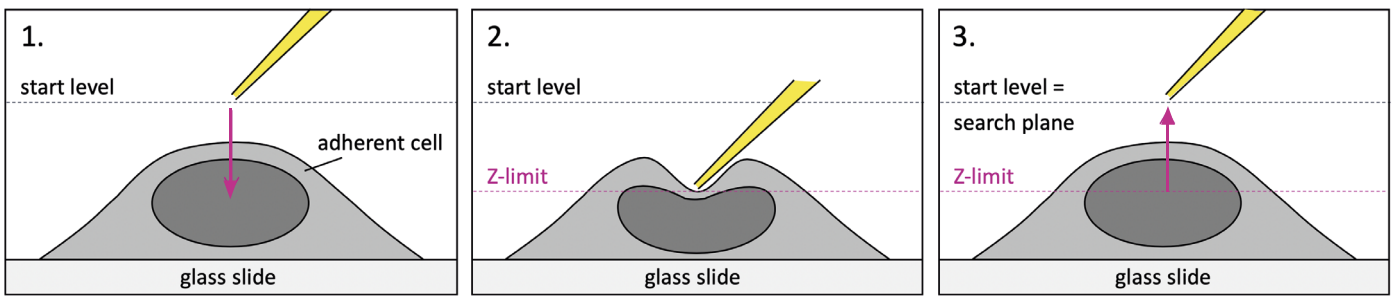


Figure 7: Setting the Z-axis limit by touching the capillary to the cell membrane and saving this height as the Z-limit.

VII. Semi-automatic Injection

A. Position the tip of the capillary in the search plane (at the start level) above the nucleus of the target cell (see Figure 8.1.). (In case of cytoplasmic injections position the tip of the capillary in the search plane above the cytoplasm near the nucleus.)

B. To initiate an injection click on the blue button of the joystick. The system now uses the current position of the capillary to calculate an axial path to the injection position at the same X and Y coordinates transposed onto the plane of the Z-limit. Thereupon the tip automatically moves to the right then quickly changes direction and moves down and to the left along a 45° axial path to a position on the Z-limit directly below your starting or targeting position (see Figure 8.2.). When it reaches this injection position the FemtoJet 4i will apply the preset injection pressure (pi) for the preset time (ti) injecting the sample into the cell (see Figure 8.3.). After the injection is complete the capillary follows a reverse axial trajectory up, them laterally back to the starting point (see Figure 8.4.).

C. When the nucleus has been successfully injected it may appear to become phase-bright relative to the cytoplasm. It may also appear to be more distinct or in-focus than before it was injected. But if it expands significantly in size it has been over-injected and the injection settings should be reduced. (When a cytoplasmic injection is successful, there will be a subtle wave of cytoplasmic disturbance around the injection point seen as a phase shift through the microscope. The nucleus may appear to momentarily become phase-dark. If the injection wave travels all the way across to the far side of the cell, the injection was too large and the injection settings should be reduced.)

D. Adjust the injection parameters ti and pi so that the cells are injected gently but do not change size, burst, or inflate. Keep in mind that you should not increase the volume of a cell's cytoplasmic or nuclear compartment by more than about 10%. Most adherent cell lines can be injected with a buffer loaded Femtotip at a pressure of 80-120 hPa, and a time of 0.2-0.4 seconds.

E. In case the semi-automated injection fails to successfully penetrate the cell membrane the height of the Z-limit can be adjusted up or down in fine increments using the Up-arrow or Down-arrow (▲▼) keys on the InjectMan 4 control board. Each click on these arrow buttons change the Z-limit by 0.25 µm.

F. If the injection is successful you can keep the adjusted injection parameters as well as the search plane and Z-limit to inject the neighbored cells at same Z-level. Position the capillary above the nucleus of another cell and inject that cell by a click on the joystick button. Try to Inject all the suitable cells in one field of view before moving the microscope stage to bring another group of cells into view. If you move to a field where the cells are out of focus you will need to refocus the microscope and set a new Z-limit.

G. Try to inject approximately one cell every 2-3 seconds. At this rate it will be possible to quickly optimize your injection parameters by constructing an expression and survival curve using different injection times (ti) and pressures (pi). Rapidly injecting cells also has the benefit of helping prevent blockage of the capillary.

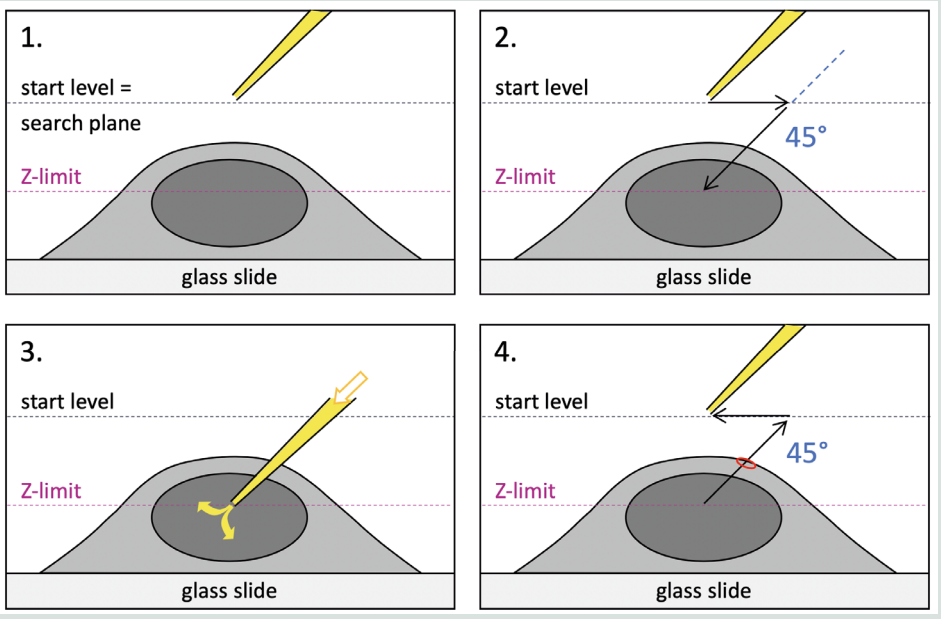


Figure 8: Semi-automated injection into an adherent cell. Injection movement of the microcapillary and injection pressure are synchronized and triggered by one click of the joystick button. This axial injection allows cells to be rapidly injected with only minimal damage to the cell membrane.

Troubleshooting

During the semi-automated injection attempt:

> **The tip of the capillary touches the cell at the Z-limit but does not inject:** Briefly press the "clean" key on the FemtoJet 4 while watching the tip through the microscope. If there is obvious flow, the tip is open. Adjust the Z-limit and try again an injection. If there is no flow, then the microcapillary may be clogged and needs to be cleared by re-using the "clean" key or must be replaced.

> **The capillary tip was tested to be open and the tip appears to touch the cell during injection but does not inject:** Move the capillary to a slightly different targeting position above the same cell compartment and try again. If that does not work, try lowering the Z-limit about a half of a micron and try again.

> **The tip of the capillary appears to touch the cell at Z-limit but does not inject the cell and leaves a mark or persistent spot on the cell:** The Z-limit may be too low. Use the Up-arrow key to raise the Z-limit by one or two µm and try again.

> **The microcapillary easily gets blocked:** Your sample preparation might still contain precipitation. Always centrifuge (for 15 min at maximum speed in a microcentrifuge) your sample immediately before the capillaries are loaded. In case of nuclear injections, try to avoid directly hitting the nucleoli as they are sticky and can clog the opening of the capillary.

Literature

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Ordering information

Description	Order no. International
InjectMan®, micromanipulator with dynamic movement control	5192 000 019
FemtoJet® 4i, programmable microinjector with integrated pressure supply, including foot control	5252 000 013
Femtotips®, injection capillary (for research use only), sterile, set of 20	5242 952 008
Microloader™, tip for filling Femtotips® and other glass microcapillaries (for research use only), 0.5 - 20 µL, 100 mm, 192 pcs. (2 racks × 96 pcs.)	5242 956 003
CellXpert®, 170 L-class CO_2 incubator	6731 000 011
Eppendorf Research® plus, single-channel pipette, variable, 0.5 – 10 µL, medium gray	3123 000 020

Interested how adherent cell injection looks like with the InjectMan 4 in action?

Click here or scan the QR-Code.

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