Microinjection of Single Cells in Culture

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Introduction

We typically microinject primary and tissue culture eukaryotic cells with cytoskeletal proteins and antibodies, but the general principles also apply to other molecules such as nucleic acids. Cells growing on 2- and in 3-dimensional substrates are amenable to microinjection. The sensitivity of a cell to microinjection depends on the cell type. Training in this type of technique relies heavily on hands-on instruction and practice, and empirically determined methods. Thus the protocols are intended to be used as initial guidelines for beginners, and include tips for those with some experience.

Methods

Cells can be microinjected directly in their culture dish, but often microinjection is combined with microscopy. In this case we plate cells on glass coverslips (12mm circles or similar for cells to be fixed, or larger circles (typically around 25mm) for cells to be viewed live on a microscope in an observation chamber. Cells tend to stick less well to glass so we usually pretreat coverslips with acid and poly-L-lysine (PLL) or polyornithine (better for some neurons).

Preparing glass coverslips

- **Acid wash coverslips.**

1. Heat coverslips in a loosely covered glass beaker in 1M HCl at 50-60oC for 4-16h.
2. Cool.
3. Wash coverslips extensively in dH2O, then ddH20.
4. Rinse coverslips in ethanol and leave to dry between a folded sheet of whatman paper (dry as separate coverslips).

5. Keep in a sterile tissue culture dish (can store for a year).

- **Coat with polyamino acid.**

1. Coat coverslips in bulk in 10-15ml 1mg/ml PLL (or 500ug/ml polyornithine), rocking or rotating for a minimum of 30 minutes in a 10 or 15cm tissue culture dish.
2. Save the polyamino acid (can reuse 3-4 times).
3. Wash the coverslips in dH2O, then ddH20 at least 5 changes in each (free polyaminoacid is cytotoxic).
4. Rinse coverslips in 100% ethanol and dry those to be used immediately on one end in an open tissue culture dish in a sterile incubator.
5. When dry, add cells.
6. Dry remaining coverslips between a folded sheet of whatman paper (dry as separate coverslips).
7. Keep in a sterile tissue culture dish (can store for a year). Do step 4 before use. Can keep 10-20ml aliquots of 1mg/ml PLL and 500ug/ml polyornithine stocks at -20 deg C. High molecular weight PLL is standard (greater than 300K), but lower molecular weight PLLs can also be tried.

- **Optional. Coat polyaminoacid/acid washed/coverslips with matrix molecules.** This helps the attachment of very poorly adherent cells (e.g. neurons), and increases the growth rate of other cell types (e.g. primary culture cells). Different extracellular matrix molecules can also change the morphology of certain cell types (e.g lamellipodia versus filopodia) (determined empirically).

1. Place polyamino acid/acid washed/coverslips into a tissue culture dish. Coat each coverslip with a drop of specific matrix molecule (about 100ul to cover 2/3rds of a 25mm coverslip-held by surface tension) from frozen stocks for a minimum of 30 minutes at room temperature, or in a 37oC incubator, or overnight at 4 deg C. Once coated the coverslips must be used fresh (within one day, or store for a maximum of one day at 4 deg C). Examples: Collagen type IV/PLL/acid washed coverslips- PC12 cells (100ug/ml collagen), somites (2mg/ml collagen). 1x matrigel/PLL/acid washed coverslips- Primary fibroblasts, neuroblastomas, fibromas, amphibian motor neurons, embryonic dorsal root ganglia. 10ug/ml laminin-polyornithine acid washed coverslips- Adult dorsal root ganglia.
2. Wash 5x with calcium and magnesium free PBS, then 1x with culture media.
3. Plate cells.

Observation Media

To observe cells on a microscope we use a media optimal for preserving cell health. Maintaining pH outside of a tissue culture incubator is most important. We reduce or omit phenol red when viewing red fluorophores in live cells. Complete media can be stored frozen in small aliquots at -20oC. We have had success with two types of observation media:

1. F12 (a low phenol red media with bicarbonate) supplemented with 10-20mM Hepes and 5-10% bovine calf sera, pen and strep.
2. DMEM, no bicarbonate, no phenol red with 10-20mM Hepes and 5-10% bovine calf sera, and pen and strep. This is a richer media and is better for more sensitive cells.
3. Some cell types e.g. neurons and primary cells have special media requirements and we also buffer their media with Hepes, and omit the bicarbonate.

Choice of Cell Type

Easy cells to microinject are flat tissue culture cells, e.g. Ptk2 cells, NRK cells, most tissue culture fibroblasts, CHO cells. Hard cells to microinject are round cells, e.g. mitotic cells and neurons, and sensitive cells, e.g. neurons and primary cells (including primary fibroblasts). Cells on a 2-dimensional substrate are more amenable to microinjection than in a 3-dimensional matrix.

Pulling Needles

We backfill microinjection needles with drawnout micropipet needles.

1. Make drawnout micropipet needles: Hold each end of a 20ul borosilicate glass micropipet between your thumb and forefinger. Position the center of the pipet horizontally over the top of an even, blue, bunsen flame (the flame should only heat the pipet in one place). Roll the pipet back and forth between your thumb and forefinger over the flame. When the glass gets red in the center and starts to get pliable, remove from the flame with both hands and immediately pull your arms apart so that the

micropipet lengthens evenly to roughly ear to ear or shoulder to shoulder width. The drawn pipet should not bend, distort or break. Immediately bring each end of the elongated glass micropipet together. This will break the micropipet in half to give two micropipet needles. Both halves can be used.

2. Protect needles: When cool place the micropipet needles horizontally in a closable container. The sharp end of the needle should not touch any surface. We keep needles in either 15cm tissue culture dishes or rectangular plastic boxes with lids. We rest the back of the needle on double sided tape that is either directly attached to the bottom of the tissue culture dish, or wrapped around a polystyrene block that we put in the plastic box. Needles can be stored for about two weeks. Significant amounts of dust and debris tend to collect on the inside of the needle after longer periods of storage.
3. Make microinjection needles in a needle puller: We like to pull borosilicate glass capillary tubing (100mm in length, 1.2mm outside diameter x 0.9mm inside diameter). The inside of each tube has an omega dot fiber for rapid and more even filling. We prefer programmable horizontal needle pullers because a particular setting tends to be more reproducible than on vertical pullers. We have had some success with vertical pullers by frequently reoptimizing settings to give the same needle. Pull a variety of needles to find the one that is best for a particular cell type/ molecule to be injected. Protect microinjection needles as for micropipet needles (step 4 above).

Filling a Microinjection Needle

1. Prepare the molecule to be microinjected: Spin 3-10ul (adequate for filling enough needles for a one day experiment) max speed in a microfuge 5-20 minutes. This removes debris/aggregates. Transfer the tube to ice. Keep the tube capped or place under a square of foil covering the width of the ice bucket.
2. Get your cells into focus on the microscope.
3. Set your microinjection system to low pressure.
4. Fill a micropipet needle: Attach the blunt end of one micropipet needle to the end of a mini-mouth pipetor. This is usually supplied with the micropipets and is made from a short piece of rubber-tubing with plastic needle holders at each end. Hold the eppendorf tube at about 45o from vertical in one hand, place the tip of the micropipet needle into the top of the solution, suck up

gently (capillary action will do most of the withdrawal). Keep the mouth-pipetor in your mouth still attached to the filled micropipet needle and return the eppendorf tube to ice.

5. Backfill the microinjection needle by inserting the filled micropipet needle into a microinjection needle. Place the tip of the micropipet into the tip of the microinjection needle. Rest the tip against the omega dot fiber. Blow out so that solution fills 1-3mm of the microinjection needle tip length. Do not overfill as this increases the chances of picking up debris and creating air bubbles. Discard any filled microinjection needle that has detectable air bubbles or is not filled to the very tip.

Microinjecting Cells

Microinjection is best done at 40x magnification.

1. Attach a filled microinjection needle to the microscope needle holder. Attach at roughly 45o to the vertical. Attach immediately after filling, and get the tip of the needle into the top of the cell media fast. This minimizes needle clogging. Position the needle in the media in the center of the brightest spot of transmitted light, while looking down on the cell media with the naked eye. This places the needle close to the field of view.
2. Look down the microscope; at this stage you will not see the needle.
3. Find the tip of the needle: There are several ways to do this all of which require looking for the shadow/image of the needle down the microscope while gradually lowering the needle to the bottom of the coverslip: remove an eyepiece and look down the occular tube; look for the needle at low power (10x or 16x) then switch to 40x; look for the needle directly at 40x (requires more skill, but some people prefer not to switch objectives). Start by moving the needle in large increments. It helps to laterally move the needle holder as this will cause the shadow of an 'invisible' needle to sweep across the field of view. The shadow will be faint at the beginning. When you see a shadow of medium intensity, switch to micromanipulation to move the needle in small increments. This minimizes trashing the needle on the bottom of the coverslip. Lower the needle until it is a tight shadow/ out of focus needle. Move to the narrowest part of the needle; this is the needle tip. Lower slowly to bring the needle tip almost into focus so that it sits a little higher than the cells. You should be able to focus clearly on the cells and see the needle tip at the

same time.
4. Increase the pressure of the injector.
5. Move the needle tip over the top of a cell.
6. Position the needle at the organelle rich region-flat region boundary of the cell. This works the best for most applications, but the nucleus can also be poked, e.g. for microinjecing DNA.
7. Lower the needle gently to just touch the plasma membrane. You should see a gentle wave pass through the cell from the site of microinjection. If nothing happens, raise the needle, increase the pressure, try again. Do not repeatedly poke the same cell; move to another cell.
8. Once the needle is flowing you may need to periodically increase the pressure to maintain an even flow.

What Helps Microinjecting a Difficult Cell?

1. Optimize media conditions (e.g. pH).
2. Optimize coating glass coverslips, e.g. some extracellular matrix molecules will somewhat flatten neuronal cell bodies (determined empirically).
3. Choice of needle, e.g. thin needles are better for round and/or sensitive cells.
4. Inject in the Z-axis instead of the more common X-Y plane.

What Helps Microinjecting a Difficult Molecule?

Hard molecules to inject are sticky molecules or protein monomers that tend to polymerize/aggregate at air-glass interfaces (e.g. actin and myosin, and to a lesser extent, tubulin).

1. Respin the molecule.
2. Try a different microinjection technique. Most needles that are pulled have an 'open tip' which is fine for injecting the majority of molecules. However aggregation etc of difficult molecules in microinjection needles can be drastically reduced by eliminating the air interface at the tip of the needle by filling 'closed tip' microinjection needles (needles with long, fine tips). However in order to microinject cells with this type of needle the microinjection needle tip must be broken after filling and finding the needle on the scope. This requires practice and a steady hand. Lower the tip very slowly onto a clear space on the coverslip. Continue lowering until a small piece of the needle tip

breaks off (about the size of 1-3 12-point periods (full-stops) at 40x magnification. Raise the needle. Move to a cell. Try injecting as above. Break more of the tip if necessary (the same needle tip can usually only be broken twice, because it gets too wide with more breaks).

Trouble Shooting

1. I can not fill the microinjection needle.
   - There are air bubbles in solution in the micropipet needle.
   - Your micropipet needle is too long.
   - The micropipet needle is not inserted into the end of the mouth-piece properly.
   - You are not blowing hard enough.
2. When I fill the microinjection needle, the solution splatters up the needle.
   - You filled the needle too much and blew out too hard.
3. The solution does not go to the end of the microinjection needle tip.
   - You did not place the micropipet needle tip far enough into the microinjection needle tip.
   - You did not fill the microinjection needle with enough solution.
4. I can not find the needle under the scope.
   - The microinjection needle was too far away from the field of view when you started.
   - You are not making enough lateral movements with the needle holder to move the needle shadow into the field of view.
   - Try another method of finding the needle (see above).
   - Do not give-up! It takes practice.
5. I find the needle, but it is trashed.
   - You lowered the needle to fast and it broke on the coverslip.
   - The needle tip was broken before you started; never attempt to use a needle tip that has touched anything even gently.
6. Nothing flows out the needle.
   - The pressure is too low.
   - Debris, air bubbles or aggregates are inhibiting flow; check this by focusing on the needle, positioned slightly off of the coverslip. Increasing the pressure may remove the block, if not start again with a fresh needle.
7. How do I know I have a flowing needle-other than by microinjecting?
   - Focus on the tip of a needle, positioned slightly off of the coverslip. You should be able to just see the flow.
8. When I pull out my microinjection needle the cell surface is pulled

with it.
- This is usually only a big problem with round cells or with cells that accumulate a lot of extracellular matrix on their cell surface.
- Try a thinner needle for round cells or adding a non-specific protein such as BSA to sticky cells.

9. I can only inject a few cells then nothing happens.
- Increase the pressure more, try again.
- There is no more solution in the microinjection needle.
- The needle picked up a patch of plasma membrane and blocked the flow.
- Debris higher up the needle was pushed by pressure into the solution in the microinjection needle.

10. My cells look funny after microinjection-when do I worry?
- A good microinjection should not significantly purturb the cell.
- Some cells may 'cringe' slightly, or retract a small region of their cell edge immediately after microinjection. This is nothing to worry about if the cell recovers promptly (say in about 5 minutes).
- Cells that undergo large, more permanent changes in morphology should be excluded from the study.

11. My cells explode.
- You injected too much and blew the cell away.
- You injected under too high pressure.
- The needle was too thin-usually a problem with round cells which require thin needles for microinjection, but will burst when the needle is too thin.

12. My cells die.
- Same as 11- but also some cells will just die even if the microinjection seemed good.
- The percentage of cells that you kill, will depend on your experience and cell type. In general an experienced microinjector will kill less than 10% of cells that are not too sensitive. As a beginner be encouraged with 15-50% survival. iii. Try injecting buffer to gain experience.

13. How do I know my cells have died?
- The nuceolus turns from black to white (phase contrast microscopy) (always occurs).
- The cell rounds-up or appears crenated (sometimes occurs).