C. elegans Microinjection

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A way of obtaining germ-line transformation of *C. elegans* is by microinjection of a desired DNA construct into the syncytial gonad (cytoplasmic continuity between the constituent cells) of the hermaphrodite worm. The DNA transformation can be passed to the next generation by extrachromosomal transformation, nonhomologous integration or homologous integration.

WORMS TO INJECT

For injection one needs to have healthy, non-starved young adult N2 worms with a few eggs. That is why it is advised to chunk the worms' plates periodically before the procedure.

The adult gonad consists of two arms, each deflexed into a U-shape. In the adult ovary, the germ-line nuclei surround a common core of cytoplasm. The distal most nuclei are mitotic, and the more proximal nuclei are meiotic. Near the bend in the gonad, plasma membranes begin to surround individual oocytes nuclei, giving rise to new oocytes (Fig.1). The microinjection of the DNA should be done in the cytoplasm of the syncytial gonad (Fig.2).

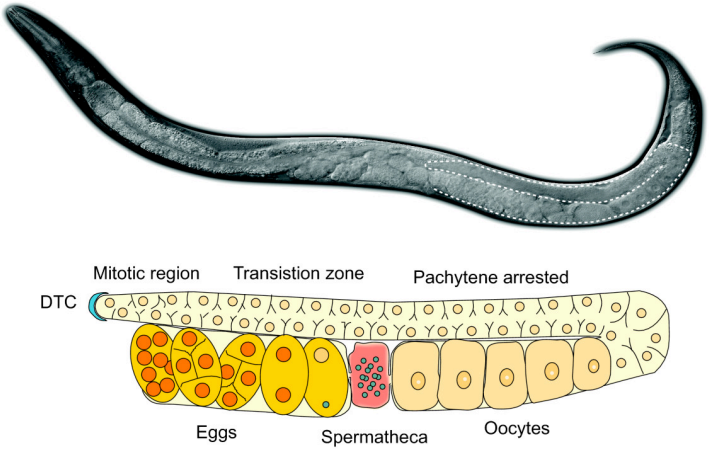


Fig.1- The *C . elegans* reproductive system. (from http://celldiv.com/content/1/1/6/figure/F2?highres=y)

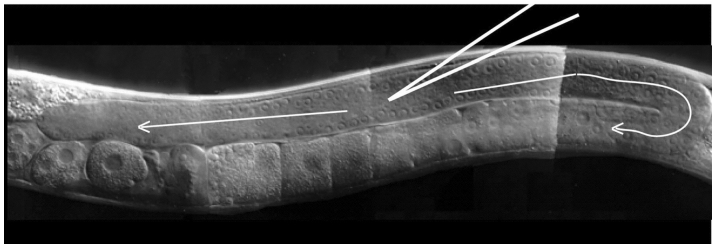


Fig.2- Microinjection of the *C. elegans* gonad (from http://www.wormbook.org/chapters/www_transformationmicroinjection/transformationmicroinjection.html)

DNA SOLUTION

For injecting worms one needs a purified DNA solution. This can be achieved by Phenol Extraction + Ethanol Purification, or by using a DNA purification column (for example a *QIAGEN QIAquick Purification Kit*, used for PCR Samples Purification Protocols). The DNA should be ressuspended in distilled water.

The DNA solution can be prepared for:

Simple Arrays: the DNA construct is mixed only with a specific marker. This will originate extra-chromosomal arrays/copies of the injected construct (if the transformation is successful), and expression of the marker in specific cells. The marker will confirm if the injections worked or not, even if the construct is not expressed. Extrachromosomal arrays are the predominant form in which a nontoxic DNA can be inherited beyond the F1 generation. The final concentration of DNA in the solution should be 200 ng/µL with, for example, 100 ng/µL of the construct DNA and 100 ng/µL of the marker (prepared in distilled water).

Complex Arrays: for this type of DNA solution, the linearized construct is mixed with *C. elegans* digested genomic DNA. For the genomic DNA preparation see http://www.biochem.northwestern.edu/ibis/morimoto/research/protocols.html. A genomic DNA sample with more than 100 ng/µL of concentration is needed. Also it has to be digested with a restriction enzyme that generates blunt ends (PvuII works well) and purified. The construct has to be linearized with an appropriate blunt end-generating enzyme. After purified, it is ready to be "mixed" with the genomic DNA. A marker may or may not be added to this solution. An appropriate ratio for the different components is: 100 ng/µL of the genomic DNA and 1 ng/µL of the linearized construct, in distilled water (and 1 ng/µL of the marker if desired).

After successful transformation, integration of the extrachromosomal arrays in the worm genome (by gamma irradiatrion) is a possibility.

Preparing the DNA

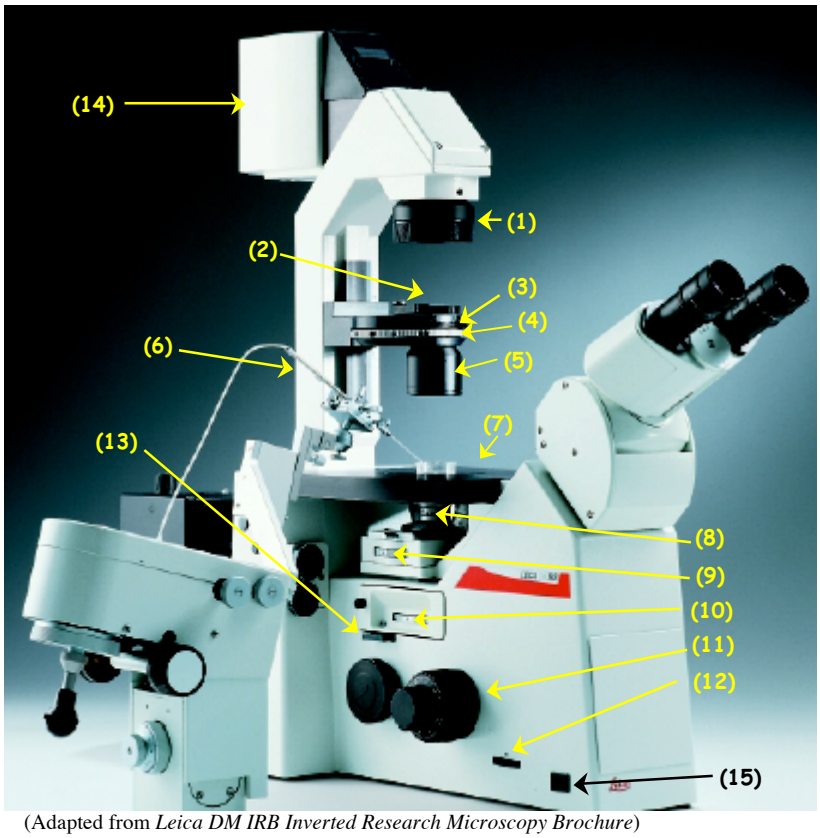
Right before use, centrifuge your DNA at full speed for about 10 minutes. This pellets any dust particles in the tube, which could plug up the needle. The spin should not be enough to pellet the DNA itself.

MATERIALS NEEDED

MICROSCOPE

To perform worm microinjections, a dissecting microscope with diffused illumination from below is used for the preparation and recovery of the animals. A high-resolution inverted microscope is used for the microinjection procedure - the *Leica DM IRB Inverted Research Microscope* works very well (Fig.3). To this equipment a microinjecting device is added – Mechanical Micromanipulator - that holds the needle and brings it into position for the injection. Before starting, the microscope has always to be adjusted and aligned. This is in fact one of the most important steps in order to be able to inject successfully.

1. Switch ON the lamp (main switch) and adjust the brightness with the dial.
2. Make sure the number on the condenser Wollaston prism matches the objective you are using (40X).
3. Make sure the DIC turret is at the correct spot (the one with DIC written on it).
4. Make sure that the dial on the objective is at zero (0).
5. Place the Ph ring all the way to the right.
6. Arrange the polarizer so that the arrow is horizontal.
7. Place a sheet of paper on the stage in order to see the focal point (light).
8. Close the field diaphragm (you should see a spot on the paper).
9. Move the condenser up and down, using the dial on the left, until the light spot becomes very sharp on the paper.
10. Open the field diaphragm slightly until you see a small circle of light.
11. Remove the paper.
12. Check to make sure the light spot is in the center of the objective. If not, manipulate the silver screws on the condenser top so that the light spot is aligned with the center of the objective.
13. Open the field diaphragm all the way.
14. Make sure the analyzer ICT/P is pushed in.



(Adapted from *Leica DM IRB Inverted Research Microscopy Brochure*)

Fig.3 - *Leica DM IRB* Inverted Microscope for Injections. (1) Transmitted light field diaphragm (2) Polarizer (3) Ph ring (4) Condenser disc (5) Condenser top (6) Injection needle (7) Stage plate (8) Multiple Objective Nosepiece (9) ICT prism adjustment (10) Fluorescence module (11) Coaxial coarse and fine drive (12) Brightness adjustment (13) Analyzer ICT/P (14) Transmitted light lamphousing (15) Main switch.

INJECTION PADS

An injection pad is a glass coverslip with a layer of 2% (w/v) agarose (in water) on the center of it. The worms that are going to be injected are transferred to these pads - the agarose layer will be responsible for immobilizing them for the procedure. There should always be a dish with injection pads near the microscope for general lab use. However, if you want to make your own injection pads, use the following procedure:

1. Prepare boiling 2% agarose in water (usually 10 mL of water with 0.2 g of agarose).
2. Place 1-4 drops (~50uL) onto the center of a long and thin glass coverslip.
3. Immediately flatten the drop with another coverslip.
4. Let this dry for a few minutes and then slide the coverslip off.
5. Label the right side of the upward-facing coverslip with an 'R'.
6. Let the slides dry for at least one day before use.

MICROINJECTION OIL

The oil is used to prevent the worm from a fast dehydration while it stands on the agarose layer. A good oil to use is the *Halocarbon Oil Series HC-700, CAS#9002-83-9*, from *Halocarbon Products Corporation*.

NEEDLES

The needles are produced from thin glass capillaries with a fine filament adhered to the internal wall (which gives the advantage of efficiently filling the tip with the liquid applied). To make needles one can use *Kwik-Fil Borosilicate Glass Capillaries (1.0 mm, World Precision Instruments, Inc.)*.

Making the Needles

1. Turn on the machine first.
2. Choose a program – Renee's program is #8 and makes very fragile but sharp needles.
3. Insert the capillary tube into the vee on the left and tighten the left knob. Unlock the capillary holding mechanism by pressing down on the metal lock above the mechanism and use the handle to push the mechanism all the way to the right.
4. Repeat this with the mechanism on the right – unlock the mechanism, pull it to the left with the handle, and tighten the knob so that both mechanisms are holding the capillary.
5. Close the cover and press "pull".
6. After a moment the capillary will be heated enough and it will be pulled into two halves. The two needles should be identical.
7. Position the needles vertically with their points downward on a piece of modeling clay. They can also be stored in a Petri dish with two strips of modeling clay to hold them.

Loading the Needles

Before loading the needle, the DNA solution has to be centrifuged for at least 10 min (13,000 rpm in the Eppendorf centrifuge) to pellet the impurities that may be present in the suspension (otherwise they would be responsible for blocking the flow in the injection needle).

1. Using the long Eppendorf brand tips, pipette about 1 μL of the centrifuged DNA solution to the very bottom of the capillary.
2. Before use, check under the dissection microscope to make sure there is liquid in the tip.
3. In the case of CRISPR lines, you need to use a mouth pipet and a hand-pulled capillary to load the needles. If you try to use an Eppendorf tip, the CRISPR solution will crystallize and clog the needle.

Mounting the Needle on the Scope

1. Turn on the Femtojet. If it has been in standby mode, hit "standby" and it will initialize.
2. Make sure that the micromanipulator knobs (the portion of the microscope that moves the needle) are all centered on their dials to allow for the widest range of motion once the needle is mounted.
3. Remove the metal rod from the needle holder by unscrewing the assembly that holds it.
4. Insert the new needle into the top portion and then put on the rubber gasket to keep the needle in place.
5. Screw back on the bottom portion of the needle holder.
6. Place the needle into its groove on the micromanipulator and tighten the screw to keep in place.
7. Use the knobs and coarse controls from the micromanipulator to place the needle's tip in the middle of the visual range at about a 45° angle in relation to the stage plate. It is important to leave it high enough so that the tip is not broken by accident.

Breaking the Needle Tip

After placing the needle in the micromanipulator, it is necessary to physically "break" its tip to allow the flow of DNA solution through it.

1. Place a square coverslip or a piece of a capillary on top of a glass slide with a drop of microinjection oil.
2. Put the slide on the microscope and bring into focus.
3. Using the fine controls, bring the needle tip to the same level of the capillary's or coverslip's side (Fig.4).
4. Briefly press the Femtojet foot pedal to make sure the needle tip is not already broken.
5. Gently move the needle so that it is just barely touching the side of the capillary.
6. Lightly tap the side of the microscope. This should be enough to break the needle tip. Note: if using Renee's needle program, you will not need to do this. Just lightly touch the coverslip to the tip of the needle.
7. Move the needle away from the coverslip or capillary and again hit the Femtojet foot pedal to make sure it broke. The needle should produce a small droplet of injection solution about 5 times wider than the needle tip on both sides (Fig.5).

   * If the droplet of injection solution is much smaller than this, try to break the tip a bit more.

   * If the droplet of injection solution is larger than this, you need to try again with another needle.

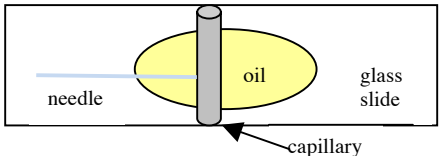


Fig.4- Position of needle for breaking.

Fig.5- Appropriate size of the injection solution droplet in comparison to the needle.

INJECTION PROCEDURE

1. Take an injection pad and put a drop of microinjection oil on it.
2. Touch a clean (flamed) pick to the oil and then pick a young N2 adult from its plate. Alternatively, just use an eyelash pick to pick the worms. Worms should be picked from areas of the plate away from the bacterial lawn, to avoid transferring large amounts of bacteria to the injection pad. Also, it is important to pick a worm with robust and well distinguishable gonads.
3. Transfer the worm to the injection pad and arrange it vertically, with the gonads you are planning to inject on the left side. Usually, the vulva is pointing to the opposite direction of the injection needle, and the two distal gonad arms are up against the body wall on the opposite side. However, occasionally the gonads will be on opposite sides or on the same side as the vulva.
4. Make sure the worm has enough oil to slow down dessication, but not enough to allow it to move around.
5. With the needle well above the slide, bring the worm into focus with the 10X objective. In oil, the gonads are visualized as two clear areas towards the anterior and posterior of the animal (Fig.6). Make sure you are focusing on the gonads and not other parts of the worm.

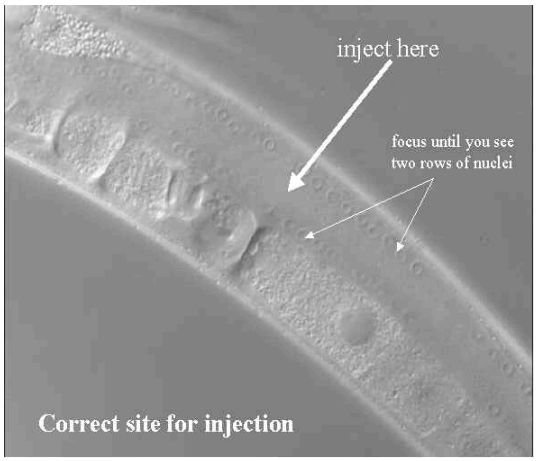


Fig.6– Picture showing where the needle should be placed to inject. (from the Chin-Sang Lab at http://130.15.90.245/photos.htm)

6. Position the worm at an orientation of 15º to 45º towards the needle, allowing a bigger path length for it inside the gonad and reducing the chances of crossing the whole body of the worm when trying to get inside (Fig.7A).
7. Bring the needle into the same level as the worm by gently lowering the needle until it comes into focus.

   * The needle should be *next* to the worm as it is lowered and not *on top* of it.
8. Change to the 40X objective and focus on the syncytial gonad arm.
9. Use the fine adjuster to move the needle up and down until the very tip is in focus with the syncytial gonad arm.

10. Very gently move the worm toward the needle and press it gently in a way that the needle moves the gonad against the body wall and ends up inside it after penetration (Fig.7C).
11. Briefly press the foot pedal to start the flow of DNA solution (a quick on-off). If the needle is really inside the gonad, it will be obvious at this point, because it swells and gets flooded. The primary goal is to put as much liquid in the gonad as possible – especially making it go around the gonad turn. However it is important to avoid the liquid blowing out the animal through the hole where the needle went in. Repeat this on the other arm of the gonad.
12. Move the worm away from the needle and lift the needle (turn counterclockwise).
13. Return to the dissecting scope to recover the injected animal.
14. Place a drop of M9 buffer on top of the worm to make it float.
15. Use the eyelash pick to lift it up and place it into another drop of M9 on a new seeded plate. This is to wash off excess oil.
16. Again, lift the worm out of the M9 and place at the edge of the bacterial field.
17. Several injected worms can be placed in the same plate. Two or three days later, the F1 (first generation) worms should be scored for the transgenic phenotype. The transgenic F1s should be singled onto individual plates to determine which ones give rise to stable transgenic lines.

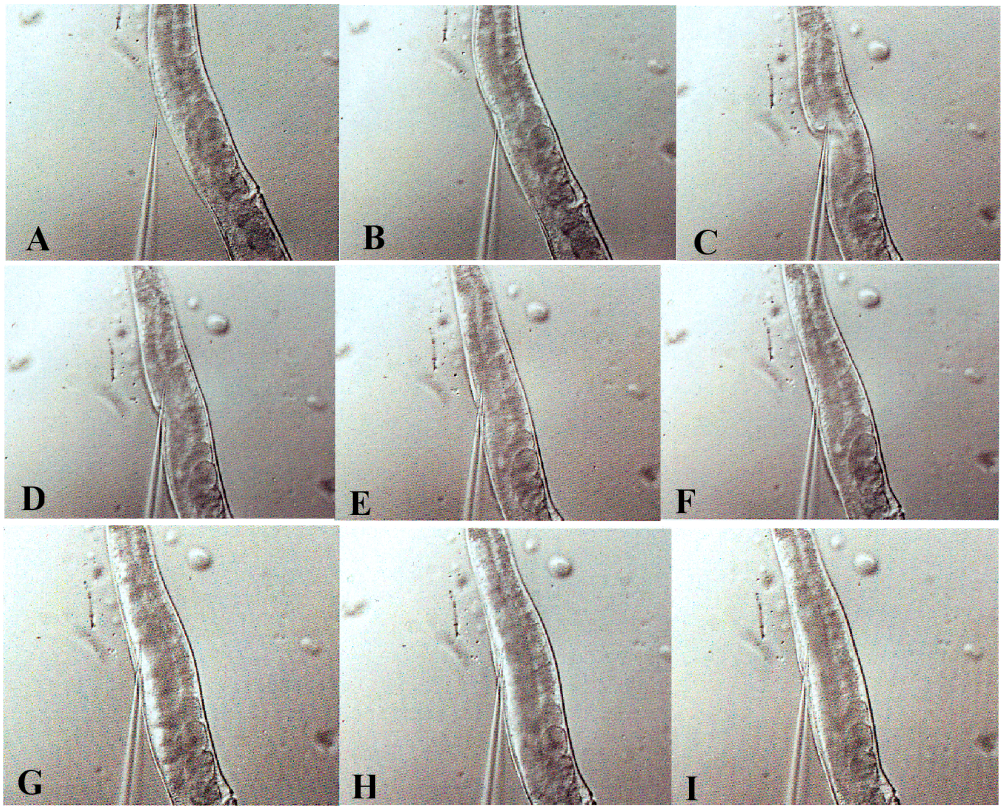


Fig.7– Different steps for microinjection of a *C.elegans* worm. A-C: The needle gets inside the gonad. D-F: Proper positioning of the needle inside the gonad. G-I: Injection of the DNA in the gonad. (Pictures adapted from the Jim Morley's movie, filmed in the Morimoto Laboratory)

REFERENCES

Michael Roella (1999) Cold Spring Harbor Laboratories Worm Course, Microinjecting Worms; Cold Spring Harbor, NY, USA.

Methods in Cell Biology. VOL. 48; *Caenorhabditis elegans*, Modern Biological Analysis of an Organism (1995) Academic Press Inc., USA. (Craig Mello and Andrew Fine, DNA Transformation chapter).