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Germline transformation of *Caenorhabditis elegans* by injection

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Abstract

Microinjection is a commonly used technique for DNA transformation in *Caenorhabditis elegans*. It is a powerful tool that links genetic and molecular analysis to phenotypic analysis. In this chapter we shall provide an overview of microinjection for germline transformation in worms. Our discussion will emphasize *C. elegans* reproductive biology, applications and protocols for carrying out microinjection in order to successfully obtain transgenic worms.

1. Introduction

1.1 Reproduction in *C. elegans*

The *C. elegans* hermaphrodite reproductive tract shows bilobed symmetry. Each lobe or gonad arm (one anterior and one posterior) is U-shaped and consists of a distal ovary, proximal oviduct, spermatheca and uterus (Fig. 1). The distal region (with respect to the uterus) of each gonad arm is a syncytium of nuclei that are eventually surrounded by membranes, and the cells thus formed give rise to the germline. Oocytes in the oviduct mature in an assembly-line fashion and enter the spermatheca, the site of fertilization in *C. elegans*. Oocytes can be fertilized by hermaphrodite-derived sperm or male-derived sperm introduced by mating. Upon fertilization, partial embryonic development occurs in the uterus, and eggs are laid before hatching. In *C. elegans*, genetic transformation is achieved by microinjection of DNA into the syncytial gonad (1,2). This ensures that the injected DNA gets incorporated into the nuclei of oocytes, and can eventually be inherited via the fertilized egg.

1.2 Microinjection, method and outcome

Kimble et al. (3) first employed transformation via microinjection in *C. elegans* where *sup-7* (*ts5*) (amber suppressor mutant) tRNA was injected into the gonad of mutant hermaphrodites. Following this, several key studies standardized injection of foreign nucleic acid sequences into the worm gonad (4-6). DNA microinjection is typically accompanied by coinjection of an easily scorable marker (Fig. 1). The choice of marker often depends on the background of the worm strain. Usually a marker whose expression will not be interfered with is chosen. One such marker used is *rol-6*, a dominant marker encoding a cuticle collagen. The mutant form causes animals to roll when they move and hence animals that are transformed can be identified easily under the dissecting scope. Another example is a strongly expressed green fluorescent protein (GFP) marker, such as *myo-3::GFP* (Fire lab vector kit) (2) which is expressed in muscle

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tissue. Although this requires the use of a fluorescence microscope it can be used reliably for detecting the presence of transgenes by detection of GFP in body wall muscles.

Injected exogenous DNA typically assembles through non-homologous recombination to form large concatemers or extra chromosomal arrays with numerous copies of the injected DNA (Fig. 1) (5). The extra-chromosomal arrays are heritable and show non-Mendelian segregation patterns. The transmission rate is positively influenced by array size (5) and can also be improved by integration into the genome (see below). In the germline, the repetitive structure of extrachromosomal arrays can cause them to be silenced (7). This problem can often be overcome by increasing the complexity of the injection mixture by including *C. elegans* or yeast genomic DNA so that tandem repeats of the extrachromosomal DNA are not formed. Injected DNA can also occasionally insert into the genome through homologous and nonhomologous integration (2). Irradiation / mutagenesis that induces chromosomal breaks in animals carrying extrachromosomal arrays (1) or micro-particle bombardment techniques can be used when integration is required (8).

1.3 Applications for microinjection

In *C. elegans*, transformation using microinjection has been used frequently for cloning by mutant rescue. A commonly used procedure for sterile mutants is to introduce the exogenous DNA into the wild type background and then cross the transgenic worms to the mutant strains. Rescue is scored as recovery of fertility in the mutant strain due to the presence of a transgene.

This method can also be used for analysis of gene expression through reporter gene assays that include using markers like GFP, lacZ or tissue- specific; ectopic and over-expression assays. Reporter genes introduced via microinjection can be used not only for the analysis of temporal and spatial expression patterns of genes but also to ascertain the conditions required for various expression patterns. Microinjection can also be used for structure-function analysis, expression of tagged proteins and RNA interference.

Once transgenic lines are established, often there is a random loss of extrachromosomal DNA during mitotic division so that animals are mosaic for a given transgene. Although this may be a problem sometimes the analysis of such mosaic animals can be used to determine the site of action for a gene (9).

2. Materials

1. Worm Pick: Platinum wire, Pasteur pipette.
2. Injection pads: 2% agarose, glass cover slip (24mm × 40mm).
3. Injection needles: borosilicate capillary tubing is commonly used (1.0 mm OD × .75 mm ID / Fiber).
4. Needle puller: Sutter Instruments, Model P-97 micropipette puller for injection needles.
5. Microscope for injection: An inverted compound microscope such as the Zeiss Axiovert equipped for Nomarski DIC microscopy or equivalent along with a micromanipulator for the needle.
6. Halocarbon oil 700 for covering worms during injection.
7. Young adult hermaphrodites (see protocol)
8. DNA for the injection mix.

9. A nitrogen tank connected to the injection rig with valves for controlling needle pressure for injection.
10. Seeded NGM plates, Dissecting microscope and 1X M-9 buffer for recovery (22mM KH_2PO_4 , 22mM Na_2HPO_4 , 85mM NaCl , 1mM MgSO_4).

3. Method

3.1 Growing and cultivating worms

1. Standard techniques and recipes for growing worms and cultivating them are used. In short, worms are grown on NGM plates seeded with the OP50 strain of bacteria (10).

3.2 Make a worm pick

1. Use thin gauge platinum wire to make a worm pick. The platinum can be flamed between uses to sterilize it, and cools rapidly.
2. Break off the long end of a Pasteur pipette to make the pick “handle.” The length of the handle depends upon individual preference.
3. Place one end of the platinum wire into the pipette and then flame this till the glass seals over the pore of the pipette, fusing the platinum wire in. Make sure to keep the pipette vertical over the flame, so that you get a straight pick, instead of a bent one.
4. Trim the platinum wire to the desired length (again, the length depends on personal choice).
5. Using a coin, flatten the free end of the pick so that it is pretty flexible.
6. Trim the flattened end of the pick using a blade so that it is quite narrow. The trimming is best carried out under a dissecting microscope.
7. The pick is now ready for use.

3.3 Prepare pads

1. Make a solution of 2% agarose in water.
2. Using a Pasteur pipette, place two separate drops of the 2% agarose (*see* Fig. 2) onto a glass cover slip (24mm × 40mm cover slips are best). Repeat for as many cover slips as the prepared solution will make.
3. Allow the agarose solution to solidify. Do not stack the cover slips.
4. Trace a circle around the agarose drop on the back surface (the surface behind the side upon which the agarose drop was placed) of the cover slip using a thin-tipped marker, and then divide this circle into quarters (*see* Fig 2). Repeat for all the cover slips.
5. Mark the front surface of the cover slip with a letter so that the front surface can be distinguished from the back.
6. Allow the cover slips to dry for a day.
7. Store the cover slips in a sealed container (so that they do not re-moisturize) on a sheet of filter paper.

3.4 Get worms ready

1. The ideal worms to inject are young adults that have already completed their first few ovulations.
2. Pick hermaphrodites at the L4 larval stage (which show a typical clear zone around the developing vulva - *see* Fig. 3) and allow them to grow overnight at 20°C.
3. These worms are ready to inject the next day.

3.5 Prepare injection mix

1. Make sure that the total concentration of all the DNA is greater than 100 µg/mL. Typically, the injection mix consists to 100 µg/mL of a marker (such as a muscle expressed GFP, or a dominant mutation that causes the worms to roll) and 10 µg/mL of the DNA of interest.
2. Centrifuge the injection mix for 10 minutes at 14,000 rpm. Carefully pipette 2-3 µL from the top into a fresh Eppendorf tube and use this for injections. Any unused DNA can be pooled back for later use. This step ensures that any fine particulate matter - which can clog the injection needle - that may be present in the injection mix is eliminated from the sample that is used to load the needle.

3.6 Clean worms

1. The hermaphrodites that are going to be used for injections must be cleansed of bacteria that can clog the injection needle.
2. To do this, place the young adults from the “Get worms ready” step onto a NGM plate that has not been seeded with bacteria.
3. As the worms crawl around on this plate, they will loose most of the bacteria that might clog the needle.
4. Keep in mind, though, that these worms are now being starved, so put the worms on these unseeded NGM plates in batches of 8-10, as they are used up for injections.

3.7 Load needle

1. There are three alternative methods to load the injection needle.
 - A. Invert the needle and place it into the DNA solution, so that the DNA is pulled up into the needle by capillary action. This process usually takes a few minutes. This method is not ideal for larger bore (0.75mm inner diameter) needles.
 - B. Heat the middle of the needle for 1-2 seconds over a flame and then place a drop of the injection mix on the non-tip end of the needle. As the heated air in the needle cools and contracts, the drop on injection mix will get sucked into the needle.
 - C. Pull a Pasteur pipette and break off the thin, pulled part in the middle. Using appropriate tubing and adapters, connect the non-pulled end of this pulled pipette to a 1 ml syringe (Fig. 4). Pull the plunger of the syringe back about half-way and then dip the tip of the pulled pipette into the injection mix. Some of the injection mix will be pulled into the pipette tip by capillary action. Once a sufficient quantity of liquid has entered the pulled pipette tip, remove it from the injection mix. Carefully thread the pipette tip into the injection needle and depress the plunger to squeeze the injection mix into

the needle. Pull the pipette tip out of the injection needle. This method is a little more complicated than the other two, but yields very good results.

2. Once the needle has been loaded, place it into the needle holder of the injection apparatus on the microscope.
3. Adjust the needle so that it makes as flat an angle as possible with the microscope stage.

3.8 Break needle

1. Break off a small part of the pulled pipette tip and place it on a 24mm × 40mm glass cover slip.
2. Overlay the pipette tip with a drop of Halocarbon 700 oil.
3. Now, focus of the edge of the pipette tip and then adjust the needle so that its tip is in the same focal plane as the edge of the pipette.
4. At this stage, ensure that the tip is intact (there should very little, or no flow of liquid when the nitrogen pump is turned on and gas is forced into the needle).
5. Very gently, move the pipette tip so that it just taps the needle. Check the flow of liquid by turning on the pump.
6. Repeat the tapping of the pipette tip onto the injection needle till it has been broken sufficiently to allow a good flow rate.
7. However, if the opening of the needle at this point is too large, it cannot be used for injections, and a new needle must be loaded and broken.
8. Once broken, prevent the injection mix from drying out by always keeping the needle dipped in a drop of oil (Halocarbon oil 700 on a glass cover slip) when it is not being used to inject a worm.

NOTE: If the tip of the needle is always too large when it is broken sufficiently for a good flow rate, there might be problem with the injection mix. Too concentrated an injection mix will often result in this problem. Try diluting the mix.

3.9 Lay worms down on pad

1. Place a drop of Halocarbon oil 700 on each of the two agar pads of one cover slip, so that the pad is completely covered by the oil.
2. Now, with the help of a dissecting microscope and the flat worm pick, pick a single worm from the unseeded NGM plate and place it on one of the unused quadrants of the agar pad.
3. Immobilize the worm by gently pushing it down onto the pad with the pick. It is sufficient for only a substantial portion of the worm to be stuck to the pad. Often, you will see about $\frac{1}{2}$ - $\frac{3}{4}$ of the worm immobilized on the pad, but the remainder will be moving. As long as the worm cannot completely come off the pad, it can be injected.
4. Make a mental note of which quadrant the worm has been placed in.
5. Transfer the cover slip with the worm onto the microscope to be used for injection.

NOTE: The agar pads hold the worm in place and start to cause desiccation. A small amount of desiccation allows for the volume of the injection without bursting the worm. However, the injection and recovery described below should be done relatively quickly to avoid lethal levels of dehydration. Over desiccation can also make injection more difficult.

3.10 Inject

1. Make sure that the injection needle is above the worm, to avoid the possibility that it will inadvertently poke or kill the worm.
2. Orient the worm so that its gonad (the gonad looks like a large sac filled with grapes) is almost parallel to the injection needle (*see* Fig. 5).
3. Lower the injection needle so that it is in the same plane as the gonad.
4. Now, carefully move the worm towards the needle so that the needle finally breaks the cuticle and enters the gonad (*see* Fig. 5).
5. Turn on the pump, so that the DNA solution in the needle is forced into the gonad. You should see the gonad filling up, like a balloon being filled with water.
6. Make sure not to inject too much DNA into the gonad, or too quickly. You do not want to see the DNA “explode” into the gonad, but rather to fill it up slowly.
7. Once you have injected the gonad, gently move the worm off the needle.
8. Raise the needle again and transfer the cover slip back to the dissecting microscope.

3.11 Recover

1. While watching under the dissecting microscope, using a 20 μ l pipette, carefully pipette one small drop of M9 onto the immobilized, recently injected worm.
2. The worm should begin rehydrating and will begin to move again.
3. Use the worm pick to carefully transfer the worm from the injection pad to a seeded NGM worm cultivation plate.
4. Make sure that each injected worm is recovered individually, so that at least three independent lines of transformants can be obtained later.

3.12 Score for transformants

1. The injected worms, if they successfully recover, will lay eggs that will hatch and be ready to score in a couple of days.
2. Score the progeny of the injected worms for the phenotype of the injection marker (e.g. GFP or rolling worms).
3. Place the transformed worms from each successfully injected worm onto a fresh, seeded NGM plate.
4. The pooled, transformed progeny from each injected animal can potentially generate an independent line of transformants.
5. Keep in mind that not all of the transformed F1 progeny from the injected worm will pass on the transgene to its progeny. Further, even the worms that do transmit the transgene through their germline will do so with differing efficiencies. The process of transformation involves a complex rearrangement of the injected DNA into a single, large, multimer of the various DNA species injected (Fig. 1). Transformation efficiency depends on the specific gene being used for transformation and the size of the recombined transgene. We have observed transmission efficiencies ranging from almost 90% to as low as 1-2%.
6. Allow the transformed F1 progeny to self-fertilize and in three days, score for stably transmitting lines.

7. For all experimental data, at least three independently derived transformed lines must be examined, to negate any artifacts from the recombination events required to form the transgene.

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Fig. 1.

A schematic overview of *C. elegans* transformation by injection and the hermaphrodite reproductive tract. Plasmids containing marker and target DNA are injected into the syncytial gonad arm. Injected DNA then forms an extrachromosomal array or minichromosome. Progeny that inherit the array display the marker phenotype.

Fig. 2. Injection pads. The injection pad consists of two drops of dried agarose. The letter on the pad (“P”) helps to indicate which side of the pad is up, since it can be hard to visualize the dried out agarose drops.

Fig. 3. Picking the right worms for injections. (A) Hermaphrodites should be picked as L4 larvae and allowed to age overnight. The arrowhead indicates the vulva clearing that characterizes L4 hermaphrodites. (B) The overnight aged worms are young adults that can be used for injections.

Fig. 4. Loading the injection needle. (A) A syringe (Grey arrowhead) is attached to the end of a pulled Pasteur pipette tip (White arrowheads - see text) using an appropriately sized adaptor (White arrow). (B) The injection solution is drawn into the pulled Pasteur pipette tip, which is then inserted into the injection needle bore. The injection solution can now be pushed into the injection needle.

Fig. 5.

Injecting the worm. (A) The needle is aligned with the immobilized worm. (B) The stage is now moved (not the needle) so that the worm is impaled on the needle and the needle enters the gonad. (C) The injection solution is now pushed into the gonad. Correctly done, the injection will result in the gonad swelling up a little bit, much like a long balloon being filled with air. (D) The stage is now moved backwards so that the needle is withdrawn from the worm, allowing the injected animal to be recovered.