Video Article

Generation of Stable Transgenic *C. elegans* Using Microinjection

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Abstract

Transgenic *Caenorhabditis elegans* can be readily created via microinjection of a DNA plasmid solution into the gonad ¹. The plasmid DNA rearranges to form extrachromosomal concatamers that are stably inherited, though not with the same efficiency as actual chromosomes ². A gene of interest is co-injected with an obvious phenotypic marker, such as *rol-6* or GFP, to allow selection of transgenic animals under a dissecting microscope. The exogenous gene may be expressed from its native promoter for cellular localization studies. Alternatively, the transgene can be driven by a different tissue-specific promoter to assess the role of the gene product in that particular cell or tissue. This technique efficiently drives gene expression in all tissues of *C. elegans* except for the germline or early embryo ³. Creation of transgenic animals is widely utilized for a range of experimental paradigms. This video demonstrates the microinjection procedure to generate transgenic worms. Furthermore, selection and maintenance of stable transgenic *C. elegans* lines is described.

Video Link

The video component of this article can be found at <http://www.jove.com/details.php?id=833>

Protocol

Expression Plasmid Construction

Two plasmids are required: one for tissue-specific expression of the gene of interest and a second as selectable transformation marker.

Experimental Plasmid

1. Select a promoter that is expressed in the tissue/cell type of interest, for example, a nerve or muscle-specific promoter. If one is to express multiple genes within the same tissue, a promoter cassette plasmid in the Gateway system (Invitrogen) can be used. This approach allows the insertion of any gene of interest downstream of the promoter by recombinational cloning ⁴. Generally, 2-5 kb of upstream sequences is sufficient to have correct and accurate expression.
2. The cDNA of the gene of interest can be cloned in two ways:
 1. Conventional cloning using restriction enzymes.
 2. For Gateway cloning, it is PCR amplified using primers that contain the Gateway *att B* recombinational sequence. The amplified cDNA is first recombined into the donor vector pDONR201 to create the entry vector and then transformed into *E. coli* strain DH5a. Following selection of successful recombinants and mini-prep isolation of DNA, the entry vector is recombined into the promoter-containing destination vector to create the expression plasmid. Details on recombinational cloning are available from either the Invitrogen Gateway manual or in Caldwell et al. ⁵.

Selectable Marker Plasmid

Examples of transgenic marker plasmids include *rol-6* or use of the body wall muscle promoter *unc-54* to drive fluorescent protein expression. These marker plasmids are usually available from within the research community upon request.

Microinjection of *C. elegans*

Preparation

1. Perform DNA isolation of the two plasmids that are to be injected. Quantitate them and mix together in a 1:1 ratio of 50 ng/μl each.
2. Prepare agar pads:
 1. Make a 2% agarose solution in water; heat or microwave until dissolved.
 2. Set out several 22 x 50 mm cover glasses on a benchtop.

3. Using a Pasteur pipette, place a drop of the melted agarose on one cover glass and immediately place a second cover glass over the drop at a 90 angle to the first. Repeat for several other cover glasses. The diameter of the flattened disc of agarose should be between 15-20 mm.
4. After the agarose has solidified (this only takes moments), remove the cover glass and allow the pad to air dry completely (several hours to overnight).
5. Store the pads in a cover glass box at room temperature.

3. Make needle loaders for DNA solution:

Needle loaders are created from 100 μ l capillary tubes that are heated in the middle over an open flame and rapidly pulled to approximately twice their length. The two halves are snapped apart once the capillary tube cools. Stockpile 10-20 needle loaders for injection. Store upright in a microcentrifuge tube rack. Use caution as to not impale oneself on the points.

4. Make microinjection needles:

The needle is made from a special capillary tube that contains an internal glass filament; this filament serves as a wick for the DNA solution. These are pulled on a needle-puller machine. We use a Narishige PP-830 puller with a heat setting of 24.8. Several needles are pulled at a time and are stored in a small plastic box. Multiple needles can be readily "mounted" on a strip of modeling clay for long-term storage.

5. Needle tip "breakers":

The tip of the microinjection needle is pulled so fine that it is actually closed at its end and should be broken open using small shards of cover glass. These are prepared by wrapping an 18 x 18 mm cover glass in a paper towel and gently applying pressure until it breaks into several pieces. Shards of a useful size are approximately 3 x 4 mm.

6. Grow wild type (N2) worms to the early adult stage for injection using standard procedures⁶. Prepare about 100 properly staged worms/construct injected.

Procedure

1. Open the main valve on a helium tank that is connected to a microinjection needle arm and holder. Close the regulator valve to allow the gas to enter the line, bringing the pressure to 35 psi. Notice that the gas can be released using a foot pedal.
2. Insert a needle loader into a standard mouth pipetter tubing (found in containers of glass capillary tubes) and draw up a small amount of the plasmid mixture (~1 μ l).
3. The needle loader is carefully placed into the back end of an injection needle and gently inserted all the way through the length of the needle until resistance is felt. Expel the DNA solution by gently blowing, then remove the loader.
4. Insert the needle into the microinjection arm, being careful to place it within the small internal rubber gasket.
5. Place a needle-breaking shard of cover glass on one edge of an agar pad. Cover the shard, as well as the whole surface of the agar pad, with halocarbon oil. Place the agar pad on the stage of an inverted microscope.
6. Position the needle in the center of the viewing field using the 4X objective and bright field illumination, but do not yet lower it into the oil. Also, position the inner edge of the shard of cover glass in the center, then focus on it (still using 4X objective). Lower the needle into the oil, bringing it into the same focal plane as the shard of cover glass. Raise the magnification by switching to the 40X objective. Refocus on the edge of the shard and reposition the tip of the needle, if necessary.
7. To open up the tip of the injection needle, GENTLY nudge the needle against the edge of the cover glass shard, while at the same time applying a short pulse of gas via the foot pedal. The needle will be sufficiently broken open when small droplets of liquid escape the end of the needle. Excess liquid flow due to too large an opening is not desirable, as it will kill the animals.
8. Remove the agar pad from the stage by raising the needle up, but do not change the X- or Y-axis position. Slide the stage out from underneath the needle, remove the agar pad and place it onto a dissecting microscope. Transfer a worm onto the pad, below the surface of the oil. If the worm wriggles, GENTLY stroke it until it contacts the agar pad. The moist worm should adhere to the dry agarose.
9. Injection
 1. Quickly place the agar pad back onto the stage, centering the worm in the field of view.
 2. Lower the needle into the same plane of focus as the worm.

3. Switch the filters to Hoffman Illumination (a type of contrast filter). This allows morphological detail of the worm to become visible. If Nomarski/DIC optics are available on the inverted scope that will work as well. Using the 40X objective, focus on the "grainy" syncytial center of the worm gonad, below the "honeycomb" pattern of the germ nuclei (choose whichever gonad is positioned on the side of the worm facing the needle).
 4. Fine tune the position of the needle until the tip is also in focus (the same plane as the gonad "graininess").
 5. Gently insert the needle into the center of the gonad and apply a brief pulse of gas pressure to expel a droplet or two of DNA into the gonad arm. You should observe a wave of liquid spread across the distal gonad. Do not assume more liquid is better, since too much liquid can flow into the proximal bend of the gonad arm, which can shut down oocyte production. If possible, depending on the position of the worm, inject the other gonad arm in the same manner.
 6. It is important to work quickly while injecting a worm, as it can easily desiccate. The decision to inject the second gonad arm is dependent upon how quickly you can re-position the needle and worm. The entire process should ideally take less than 1-2 minutes.
10. Remove the cover glass from the stage and place it on a dissecting scope. Apply 1µl of M9 buffer (22mM KH₂PO₄, 42mM Na₂HPO₄, 85mM NaCl, 1mM MgSO₄) directly onto the injected worm (this might entail submerging the pipette tip below the surface of the halocarbon oil). The buffer should rehydrate the worm; it will then float off the agarose pad surface. Transfer the worm to a regular plate using a worm pick. The agarose pad can be reused several more times until it has become too saturated with buffer and the worms no longer adhere.

Transgenic selection

1. Injected worms, the P₀ generation, are transferred to individual fresh, bacterially seeded plates (60 mm) and allowed to reproduce. Typically, injected animals are incubated at 20°C for 2-3 days until the offspring appear.
2. The F₁ offspring are observed for evidence of the transgenic marker (such as fluorescence) using a fluorescent stereomicroscope. Each fluorescent, carrier F₁, animal is separately cloned onto a new plate (since offspring from each F₁ are considered a distinct line). The F₁ hermaphrodites are allowed to reproduce. Again, this is typically performed at 20°C for 2-3 days until the offspring appear.
3. The F₂ generation is observed for transgenic animals as well. F₂ animals that have inherited and express the transgenic array are considered a "stable" line.

NOTE: At the F₁ stage there may be many transgenic animals, but they are not stable. The majority of F₁ transgenic animals will not be propagated into F₂ stable lines.

NOTE: To control for the variability in the gene copy number, generate a minimum of three independent stable lines per construct injected.

4. Each independent stable line should be maintained as a separate line of worms. Each line can be propagated by transferring several transgenic animals to a new plate at each generation; this must be performed using a fluorescent stereomicroscope if the selectable marker is fluorescent.

Discussion

When doing this procedure, it is important to remember to:

- inject directly into the center of the gonad
- not inject too much liquid
- work quickly to prevent dessication.

If you run into problems with the needle, such as it is broken too large, it is best to remake a fresh needle, rather than trying to inject with a less than ideal needle.

The generation of transgenic worms has many applications such as:

- identification of mutant genes, in method called transformation rescue
- mapping of protein domains through expression of truncated proteins
- characterization of the role of a gene in cellular and disease processes.

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