Microinjections in *Nothobranchius furzeri*

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Equipment Used:

1. Narishige Puller (PC-100)
2. Narishige Microgrinder (EG-401)
3. Warner Instruments Pico-Liter Injector (PLI-100A) connected to nitrogen line
4. Narishige Micromanipulator (M-152)
5. VWR microscope (89404-478)

Reagents and Other Tools:

6. Borosilicate Glass (O.D: 1.0mm, I.D: 0.58mm, L: 10cm) *
    a. Non-filamented: Sutter Instruments (available from #GB100-58-10, VWR#100357-958)
    b. Filamented: Sutter Instruments (available from #GBF100-58-10, VWR#100357-986)
7. Exel International Brand Quality Luer Slip Syringes, Air-Tite Products (sterile, disposable, 1mL, VWR#76290-424)
8. MicroFil™ Pipette Filler, World Precision Instruments (Gauge:28, L:67mm, I.D:250µm, O.D:350µm, VWR#MSPP-MF28G675)
9. Killifish embryo solution
    c. Ringer's solution ¼ strength tablet (Sigma-Aldrich#96724-100TAB)
    d. Quick Recipe: Add 2 Ringers tabs to 1L dH_2O. Autoclave. Add 100 uL of methylene blue solution to 1L once cooled. If some water evaporated during autoclave, make sure you replace that with autoclaved dH_2O to bring back to 1L mark.
10. Killifish egg agarose mold with individual wells **
11. Certified Molecular Biology Agarose (BioRad#1613102)
12. IBI Certified Molecular Biology Agarose Low Melt (VWR#IB70050)
13. VWR petri dish, large 100 x 15 mm (VWR#89038-968)
14. Mineral oil
15. Parafilm (VWR#52858-076)
16. Calibration glass slide (preferably with a silicone O-ring)
17. Phenol red solution, 0.5%, sterile-filtered (Sigma-Aldrich#P0290-100ML)
18. Microcentrifuge tubes, 2.0mL, RNase/DNase-Free (VWR#87003-300)
19. Plastic pipettes, 3mL, non-sterile
20. dH_2O squeeze bottle
21. Needle disposal glass bin
22. Thick capillary tubes with polished closed end (KIMAX-51, 1.5-1.8x99MM, #34505)
23. Analytical scale, d=0.1mg (VWR#75802-858)
24. KCl (2M)
25. Nuclease-free water
26. Injection components (for transgenesis or CRISPR)

***** Filamentation allows for easier backfilling.

** 3D printable blueprint available upon request.

Equipment Usage Instructions:

Three main conditions alter the injection volume, 1) P_{inject}, 2) injection time, and 3) the needle tip shape and length.

1. Needle puller (NARISHIGE PC-100)

This needle puller can create needles with a tip ranging from 1-10µm. It is capable of one-stage and two-stage pulls and is adjustable for pulling force and pulling temperature. Decreasing weight load and increasing the pulling temperature will result in longer needles. Likewise, increasing weight load and decreasing the pulling temperature will result in shorter needles. Each needle should be calibrated to determine the injection volume (*see 'Mini-Protocols' section*). This is necessary to create reproducible microinjections.

2. Micro Grinder (NARISHIGE EG-401)

Needles can be grinded at an angle for a sharper tip. This can allow for greater needle customization, specifically for injecting though chorions that are particularly tough or weak.

3. Pico-Liter Injector (WARNER PLI-100A)

$P_{balance}$: The pressure used to counteract capillary action and should be high enough to where liquid is never going up the needle. This needs to be recalibrated each time a new needle design is being used.

P_{out}: The active pressure leaving the needle.

P_{inject}: The pressure to inject. This alters the injection volume.

P_{clear}: This is the total pressure of nitrogen coming into the machine. This should be lower than 50PSI. An acceptable setting is anywhere between 42-45PSI.

4. Micromanipulator (NARISHIGE M-152) with magnet base (TRITECH RESEARCH GJ-1)

Can be adjusted for the user to comfortably reach each egg. The easiest place to start for adjusting the arm is with all the settings at 0.

For *Nothobranchius furzeri* injections, our settings are:

Needle Puller:

PULL: 2-stage pull, DIST: 6mm/3mm, WEIGHT: 2 Light + 2 Heavy, TEMP: 65˚C/60˚C

Microgrinder:

If egg chorions are weakened by placing in fridge (*see 'Injection Protocol' section*), then end micro grinding is not necessary. Otherwise, a 25° grinding angle works well to penetrate the chorion.

Pico-Liter Injector:

These settings are adjusted depending on the individual needle; however, these are the settings we use most of the time with the specific needle design described above.

$P_{balance}$: 1.6 PSI, P_{inject}: 8 PSI, Injection time: 0.1 seconds

Micromanipulator:

The needle is best positioned at a ~65˚ angle to the plate bottom. Looking from the microscope, the needle should be positioned to enter the egg from the NE, in respect to the center of the egg.

Injection Protocol:

1. Place sand dishes in tanks and allow breeding pairs to mate for 3-4 hours.
    a. Sand can be placed overnight when the lights are off and collected in the morning.
2. Bring agarose plates to room temperature. If they are fresh and warm, let them cool. If they were stored in the fridge (at 4˚C) place them in the room to warm up.
    a. Plates are 1.5% agarose (*see 'Mini-Protocols' section*).
    b. If you are not using a plate that holds each egg individually and instead have a trench mold, consider using a low melting agarose cover (*see 'Mini-Protocols' section*).
3. Make fresh injection needles.
4. Collect fish eggs and bring to lab.
5. Sort through eggs for healthy eggs in 1-2 cell stage.
    a. 2-cell stage eggs are easier to penetrate and inject.
6. Place good eggs onto agarose plate mold. Pour just enough embryo solution to cover each egg in a well.
7. Place eggs into 4˚C for 10 minutes.
8. Get injection mixture together and keep on ice.
    a. Only fill needles one at a time, before injecting.
9. Prepare the pico-liter injector. Turn on the Pico-Liter Injector first! Then open the knob for the nitrogen tank. You do not need to re-adjust the regulator.
    a. The nitrogen tank regulator has 2 pressure gauges and a knob for each dial. The pressure gauge on the right shows what is leaving the tank and going into the regulator. It will vary depending on how full the nitrogen tank is. The pressure gauge on the left shows what is leaving the regulator and going into the pico-liter injector. It should be close to 40-45 psi. You can confirm this by checking the 'P_{clear}' setting on the pico-liter injector.
10. Install a needle into the injection arm and position the arm in a comfortable way.
11. Remove eggs from fridge and begin injections.
    a. Manipulate eggs as you go with a polished end capillary tube. The cell is best positioned facing up in the NE direction from the middle of the egg.
    b. Make more needles as you need them to keep their tips sharp.
    c. Backfill needles as you need them, with the syringe filler.
12. After injections are complete, close the knob to the nitrogen tank first! Do not turn off the Pico-Liter Injector until the 'P_{clear}' setting reaches 0. It will take about ½ hour before it reaches 0. If you are unsure or must leave lab, leave the Pico-Liter Injector on overnight and turn it off the next morning.
13. Keep eggs in agarose mold, and cover with methylene blue embryo solution. Store eggs in incubator (26-28˚C)* and check after 24 hours. Gently remove from agarose mold after 24-48 hours. Store in methylene blue solution until eyes develop, then place on coconut fiber.
    a. Expect a 50-90% survival rate as you gain experience.

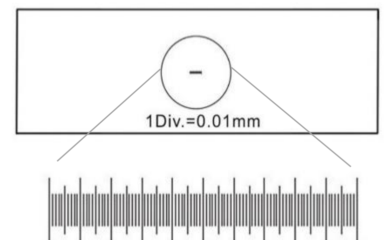
***** Storing at a lower temperature reduces fungal spread but also reduces development rate. We found the best solution is to store eggs for their first 2-3 days at a lower temperature, then raise the temperature by 1˚C every day until eggs can be stored at the max temperature.

Troubleshooting and Tips:

1. Fish are not laying enough eggs
    a. Allow naïve individuals to breed for 1 week before collecting eggs for microinjections. Young fish need some experience before high quality, fertilized eggs are produced.
    b. Give breeding pairs a break of a day or two between breeding sessions.
    c. Remove all sand that might accumulate in the housing tank outside of the breeding dish.
    d. Acclimate the fish to breed at the same time of day. They are more likely to mate after a recent feeding or water change, so try to have a consistent schedule based on their feeding times and ensure water quality is within healthy parameters.
    e. Make sure breeding sand is autoclaved at least once a week to reduce chances of harmful microorganism damaging or infecting the eggs.
2. Needle is shifting and not stable when going to inject.
    a. The needle should be firmly installed within the injection apparatus. Only 25% of the needle should be hanging out of the injection arm. If it is not stable, replace the O-ring within the injection arm.
3. Injecting but no solution is coming out of the needle.
    a. Check to make sure the needle opening is unobstructed. You can test it by injecting once onto a Kimwipe, or by hitting the red button 'CLEAR'. If nothing comes out, then the needle is not open. Gently rub the end against a Kimwipe until it is open or break it against the chorion of an egg.
4. Eggs are getting stuck on the needle every time I inject.
    a. The best way to avoid this is to use an agarose mold that creates individual wells for each egg. The other option is to change the angle that the needle is penetrating the egg.
5. Eggs are bursting when injected.
    a. The injection amount is too high, your needle aperture is too large, or the egg has a fungal or other infection that is making it particularly weak and unable to withstand injections.
6. Too much liquid is seeping out of the needle in between injections.
    a. Either your needle aperture is too large, your $P_{balance}$ is set too high, or your plate has been filled with too much methylene blue solution.
7. While injecting the egg fluids are taken up into the needle.
    a. If your $P_{balance}$ was not problematic when you first started injecting, it is possible that your needle tip has broken and the aperture is too large. Change out your needle.
8. My needle is breaking every other egg.
    a. That is normal and will get better with practice.
9. Having a hard time breaking tip of needle on first egg.
    a. Go slow and position the needle straight and head on. Do not let the tip bend when you go to break it against the chorion. Make sure the needle is fully stable within the injection arm.

Mini-Protocols:

1. **Making a 1.5% agarose injection mold plate:**
    a. Add 50mL of dH_2O to a small beaker on a hot plate.
    b. Add a small stir bar. Turn on stirrer to low.
    c. Measure out 0.75g agarose.
    d. Begin to heat on medium, and sprinkle in the agarose. It should not clump. Cover the top while heating.
    e. Once the solution turns from opaque to clear with slight bubbling, turn off heat and stir bar. Pull it off the heat onto the workbench, and let it sit for a few seconds until most of the bubbles are gone.
    f. Slowly pour into 2, 90mm petri dishes. Let sit or jiggle for a few seconds to remove any tiny bubbles.
    g. Slowly place the casting mold onto the gel.
    h. Let cool either at room temperature, or in the fridge. They can also be labelled and sealed with parafilm and stored for up to 1 week.
2. **Making a 1% low-melting agarose cover:**
    a. Add 15mL of dH_2O to a small beaker on a hot plate.
    b. Add a small stir bar. Turn on stirrer to low.
    c. Measure out 0.15g of low-melt agarose.
    d. Begin to heat on medium-low, and sprinkle in the agarose. It should not clump. Cover the top while heating.
    e. Once the solution turns clear, turn off heat and stir bar. Pull it off the heat onto the workbench.
    f. Let the gel cool to 28°C before pouring onto eggs. You might not need to use all the agarose. You just need enough to cover the eggs.
3. **Making microneedles:**
    a. *N. furzeri* settings:
        i. Puller: 2-stage pull, DIST: 6mm/3mm, WEIGHT: 2L/2H, TEMP: 65˚C/60˚C
        ii. Grind: 25˚ (optional)
4. **Calibrating microneedles:**
    a. After a microneedle is made, grab ahold of a calibration slide. The calibration slide will have a tiny ruler, with lines indicating various lengths of measurement. For example, our slide has this scale 12 small lines or ~2 big lines = 1 nanoLiter.



    b. Place a drop of mineral oil on the slide, fill your microneedle with a dyed solution and inject into the mineral oil. The diameter of the droplet dispensed can be measured against the ruler. This will give you an approximate injection volume.
    c. This exercise is also great for practicing your control over the $P_{balance}$ setting. Injecting in the mineral oil is typically harder than injecting in an embryo, so beginners might benefit from practicing in mineral oil.

Transgenesis:

Here we will make killifish embryos that express EGFP. We have two main injection components: the mRNA (transposase, pCS-TP) and the DNA (EGFP, pT2AL200R150G), both of which we isolated from plasmids.

Reference concentrations:

References:	mRNA (ng/µL):	DNA (ng/µL):
(Harel et al., 2016)	30	40
(Hartmann and Englert, 2012)	25	25
(Valenzano et al., 2011)	15	15

Calculating concentrations:

$$amount\ (\mu L) \times \frac{concentration\ (\mu g)}{total\ volume\ (\mu L)} \times 1000 = final\ concentration\ {}^{ng}/_{\mu L}$$

Component:	Concentration:	Amount:	Recommended Final Concentration (when total volume is 35 µL):
mRNA	0.5-1 µg/µL	1-2 µL	30 ng/µL (Acceptable range: 14-57 ng/µL)
DNA/plasmid	0.75-1.5 µg/µL	1-2 µL	40 ng/µL (Acceptable range: 21-86 ng/µL)

Example step-by-step calculation:

I. Calculate total mRNA:

$$2\ \mu\text{L (amount)} \times \frac{1\mu\text{g}}{\mu\text{L}}\text{(concentration)} = 2\ \mu\text{g of mRNA in solution}$$

II. Calculate mRNA concentration:

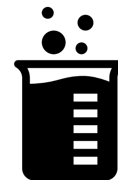
$$2\ \mu\text{g mRNA} \times \frac{1}{35\ \mu\text{L of total volume}} = \frac{0.057\ \mu\text{g}}{1\ \mu\text{L}}\ \text{mRNA final concentration}$$

III. Convert µg to ng:

$$\frac{0.057\ \mu\text{g}}{1\ \mu\text{L}} \times \frac{1000\ \text{ng}}{1\ \mu\text{g}} = 57\ \text{ng}/\mu\text{L}$$

Recipe:

- **2** µL RNA (0.6 µg/µL*)
- **2** µL DNA (0.9 µg/µL*)
- **7** µL KCl (2M)
- **7** µL Phenol-red (0.5%, filter sterilized)
- **17** µL Nuclease-free water



TOTAL: 35 µL injection solution

*These are example concentrations that we calculated using a Qubit 4.