Microinjection Techniques: Injecting through the chorion

I. Introduction

To investigate the role of a gene during development, overexpression or misexpression of your gene of interest is a fast assay. You can inject mRNA or DNA of the wild-type gene or a constitutively active form of the gene to investigate its gain-of-function phenotype. To investigate its pseudo "loss-of-function", you can inject various mutant forms such as dominant-negatives. You can also "knock down" a gene using a morpholino antisense oligo (Nasevicius, A. and Ekker, S.C., 2000. Effective targeted gene 'knockdown' in zebrafish. Nat Genet. Vol. 26, pp. 216-20).

In this lab, we will assay the effects of overexpression of BMP pathway components by injecting mRNA into the yolk through the chorion. We will also inject a *smad5* mutant, *somitabun*, with wild type *smad5* mRNA to rescue the mutant phenotype. We will inject morpholino oligos into the yolk to knockdown components of the BMP pathway and rescue these "morphants" by double injection of mRNA encoding the component, but not containing the morpholino binding site.

II. Materials

A. mRNA encoding Your Favorite Gene

B. 0.5% phenol red in DPBS (Sigma, cat. no. P-0290)

C. 0.1M KCl

D. 1X E3 medium
   5 mM NaCl
   0.17 mM KCl
   0.33 mM CaCl2
   0.33 mM MgSO4
   We make up the E3 as a 60X stock solution. When making the 1X E3, add 20 microliters 0.03 M Methylene Blue per 1L of 1X E3 (prevents fungal growth).

E. E3 medium/1% agarose dishes with furrows to hold embryos in place (described in Zebrafish Book). Molds to make injection wells (6-well zebrafish injection molds, I-An Manufacturing, 8 Jan Sebastian Drive, Unit #16, Sandwich, MA 02563, 508-888-2321).

F. 1mm capillary tubes (World Preciscion Instruments, Sarasota, FL: cat.no TW 100-F)

G. Pipetman and sequencing gel loading tips (Continental Lab Products, cat.no. 2029

H. Dumont #5 watchmaker forceps (older ones are not as sharp and will be less damaging to embryos)

I. 1 x 0.01 mm Stage micrometer for droplet calibration (Fisher, cat. no. 12-561-SM1)

J. Halocarbon Oil Series 27 (Sigma, cat. no. H8773)

K. Glass or plastic pipet for embryo transfer

L. 10 cm petri dishes for embryos after injection

III. Preparation of RNA and Needles

A. **RNA synthesis:** We use the Ambion mMessage kit to synthesize *in vitro* capped mRNA. This RNA can then be diluted in 0.1 M KCl and phenol red. We generally dilute the RNA to a 1 in 5 dilution, which can then be further diluted to titrate the RNA. Morpholinos are treated differently. Please see section VI for details.

B. **RNA titration:** Every batch of *in vitro* transcribed mRNA must be titrated, even if the concentration looks similar on a gel or spectrophotometricly. The activity of the RNA can vary due to different efficiencies of the capping reaction. If you intend to use a particular RNA a lot, it is a good idea to transcribe several batches at once and mix them together, then titrate the mixture. Aliquot the RNA in small quantities (3-5 µl) and store at -80^0C. Frequent freeze/thaw can result in mRNA degradation and loss of activity.

C. **Injection needles:** We make our needles from capillaries with an internal filament. The filament allows for efficient wicking of the nucleic acid solution to the tip of the needle following backfilling (see below). Establishing the correct settings on the needle puller is important in making a good needle. Needles with a long shank tend to break more easily and can bend on, rather than penetrate, the chorion. Needles with a short shank are sturdier and don't break as easily, but can be damaging to the embryos as they tend to thicken quite quickly. Needles somewhere in between tend to give the best results. **A GOOD NEEDLE IS KEY TO THIS TECHNIQUE.**

IV. Preparing to Inject

A. **Switch on the microinjector and open the pressure valve.** Set the injection pressure between 10 and 20 psi. The balance pressure should be 0.2-0.4 psi to prevent medium from flowing back into the needle and diluting your RNA or introducing contamination.

B. **Backfilling the needle**

1. Spin RNA/DNA/morpholino before loading it into the needle, so particles that could clog the needle are in a pellet
2. One can use sequencing gel loading tips (that have a long thin tip) to load the RNA. These tips fit inside the back of the needle, and the RNA flows to the tip by capillary action.

3. Place the needle on a vertical mound of clay for loading. By not holding the needle, you prevent the air between the RNA and the closed tip from expanding and expelling your RNA from the back of the needle. It also helps to load at least 1 µl of RNA. It is important to keep the meniscus of the liquid in the needle above the tapering of the needle. This prevents inflow of the E3 media by capillary action.
4. When the RNA has migrated to the tip, introduce the needle into the needle holder on the micromanipulator.

C. **Breaking the needle**

1. Needles can be broken either in the air or in the E3 medium.
2. Using the highest magnification on your dissecting scope, gently scrape the tip of the needle with clean forceps. Only a very small amount of the tip needs to be broken.
3. Press the injection pedal to be sure that the tip has been broken and RNA can flow out of the needle

D. **Calibrating the needle to estimate the amount injecting**

1. Clean the calibration micrometer slide with EtOH and put a drop of halocarbon oil on it.
2. Focus the microscope at 2X magnification on the level of the calibration slide to see the scale, which divides 1 mm into 100 units.
    a. For the best distribution of RNA in the 1-cell embryo, a volume of 2 nl should be used.
    b. For single blastomere injections at later stages (4, 8, 16 cell, etc), the injection volume should be decreased in a similar manner. These cells are smaller and cannot take up such a large volume without being damaged.
3. Press the footpedal to observe the size of the drop in the oil. The drop should hang in the oil to form a perfect sphere. If the drop falls and spreads out on the slide, use a larger volume of oil to create a thicker layer. A slight increase in diameter changes the volume by the third power. Thus, a 0.13 mm sphere already has twice the volume! (Volume of a sphere = $4/3\pi r^3$). See attached calibration table for a quick estimate of how droplet size corresponds to volume.
4. By changing the injection pressure or time, you can change the volume. If you are having trouble obtaining larger volumes, you can clip a little more off the end of the needle and then recalibrate the needle. Remember, needles that are too thick (too short) damage the embryo and reduce survival!

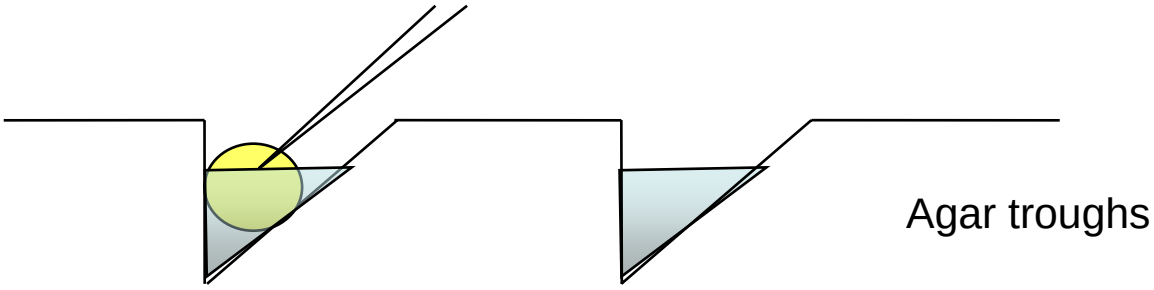
E. **Submerge** the tip of your needle in to the E3 medium in the injection plate to prevent drying out of the RNA at the tip and clogging the needle. You are now ready to inject!

V. Injection

A. **Magnification:** For injection, change the setting on your microscope to 1.2X or 1.6X magnification

B. **Organize embryos**

1. Move your needle out of the injection plate and arrange your collected embryos in the furrows of the agar with the transfer pipette and probe.
2. Remove nearly all the E3 medium for injection. THIS IS VERY IMPORTANT. This provides surface tension that prevents the embryo/chorion from sticking to the needle when trying to remove it after injection.



C. **Injection into the yolk**

1. Carefully extend the needle through the chorion and into the yolk and press the injection pedal. If the needle is within the yolk, the injected RNA/phenol red will not diffuse immediately. If the needle has extended through the yolk and lies outside of the embryo, the phenol red will diffuse immediately. If the injection is successful, you will see a red spot in the yolk or cytoplasm.
2. RNA is most active when injected directly into the embryonic cell instead of the yolk. However, this requires careful orientation of the embryos, and is more time consuming. The RNA that has been injected into the yolk will move into the cytoplasm via cytoplasmic streaming.
3. Once you have mastered yolk injections, you may want to try injecting into the cell. The cell membrane is tougher than the yolk membrane, so we typically enter the needle through the yolk and extend it into the cytoplasm. To do this, you should orient the embryo in the furrow such that the animal pole is opposite the needle.

VI. Morpholino Injection

Morpholinos designed to the 5' UTR or early coding sequences act by blocking translation. Morpholinos can also be designed to overlap splice sites to block splicing (Draper, B. W., Morcos, P. A. & Kimmel, C. B., 2001. Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. Genesis.Vol. **30**, pp. 154-6).

The actual injection technique for morpholinos is the same as that for RNA injection into the yolk. However, there are some differences in preparation that we will discuss in this section.

A. **Preparation**

1. **Do not dilute morpholinos in KCl.**

a. The manufacturer (Gene-Tools) suggests that morpholino powder be resuspended in sterile water or 1X Danieau solution (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES pH 7.6).
b. We resuspend our morpholinos in sterile Millli-Q water to a concentration of 65 mg/ml (usually around 40 microliters of water).

2. Once the stock solution is made, morpholinos are diluted in phenol red and 1X Danieau. Do not attempt to inject the aqueous morpholino solution, as this can be toxic to the embryo.
3. Morpholinos must also be titrated to determine the lowest possible amount that is necessary to elicit a specific phenotype. At higher amounts (above 4-10 ng, depending on the MO) morpholinos often cause non-specific effects, such as brain or general cell death.

B. **How can you show that a phenotype is the result of knocking down ONLY your gene-of-interest?**

1. Common non-specific effects of morpholinos are a general delay in development and widespread cell death. Even low amounts of morpholino delay development, so it is important to stage-match control embryos, rather than age-match them. A second non-specific effect is cell death, which can be low and transient, frequently restricted to the brain, or widespread causing early lethality.
2. You can show that the phenotype is specific by rescuing the phenotype with wild-type mRNA of your gene-of-interest. However, morpholinos are frequently made to the 5' UTR of the gene, and this sequence may be included in your *in vitro* transcribed mRNA. Thus the morpholino will inhibit the injected RNA as well. To circumvent this problem, mRNA of an orthologue from a different species can be used or a construct can be made with a different 5'UTR sequence. We will be rescuing morpholino phenotypes with the corresponding mRNA from Xenopus or mouse.
3. To do the rescue experiment in a controlled manner, each embryo is injected twice, first with the morpholino and then with the RNA. In this way, one can be certain that the amount of morpholino injected is at the appropriate level to give the phenotype and that the mRNA truly does rescue the morphant phenotype. Two approaches can be taken.
    a. Inject the morpholino WITHOUT phenol red into all embryos. Then go back and inject every other embryo with RNA that has been diluted with KCl and phenol red. After finishing the injections, you can then go back and pick out the embryos which have been injected with RNA by looking for the residual phenol red spot in the yolk.
    b. Alternatively, inject the morpholino WITH phenol red first. Remove all the injected embryos. Place 1/2 back in the trough and inject with RNA containing phenol red. This is what we will do. Compare phenotype of morpholino-injected embryos alone versus morpholino + mRNA-injected embryos.
4. In some cases, injection of the wild type mRNA may degrade too early to rescue the late manifesting morphant or may cause gain-of-function defects even at low doses. The next best control is to design a second morpholino, not overlapping with the first. If it elicits the same phenotype as the first, then this provides support that the defect is specific to the gene of interest.
5. For translation blocking morpholinos, an additional control should be performed to show that translation of the gene of interest is indeed affected. An antibody to the protein can be used to show that translation is blocked by Western blotting. If an antibody is not available, then the typical control is to show that

expression of a tagged version of the protein (e.g. myc or Flag tagged), when injected as mRNA into the embryo, is blocked on a Western blot.

6. For splice blocking morpholinos, RT-PCR should be performed to show that splicing is altered. It is important to sequence the mis-spliced mRNA to be certain that the ORF is sufficiently altered to cause loss of protein function.

VII. Tips and tricks

A. Avoid fast movements when you handle something under the microscope.

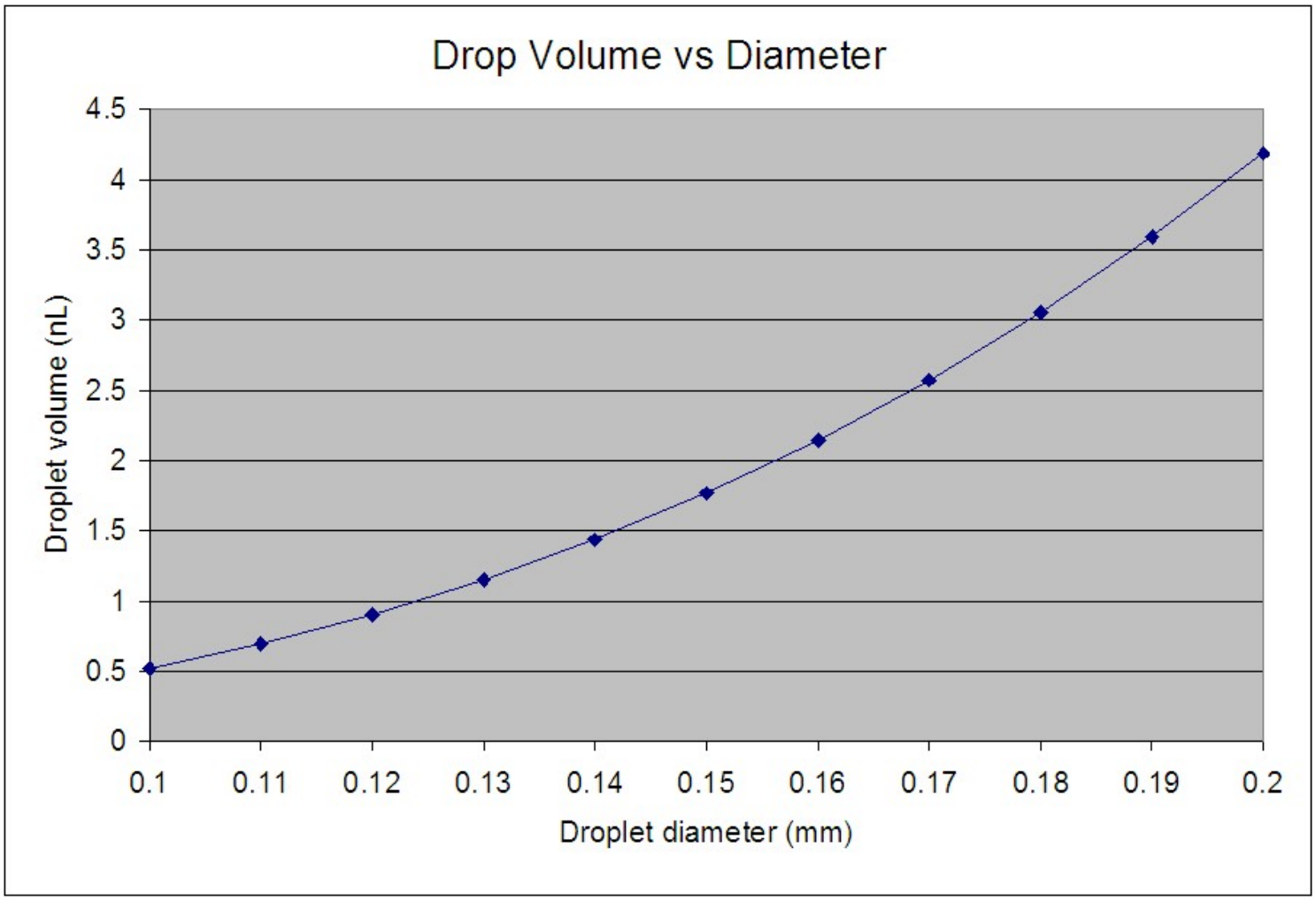
B. If the needle gets plugged there are several options, which may help:

1. Press the clear button.
2. Scratch very carefully the needle over parafilm or your forceps to try to remove visible debris at the tip.
3. The last option is to rebreak the tip and recalibrate the needle.

C. If you inject increasing amounts of RNA, start with the lowest. You can use the same needle for higher concentrations. Inject increasing volumes up or expel lower concentration RNA and then backfill with higher concentration RNA.

D. Store your needle with RNA loaded for future use at 4^0C in a sealed humid chamber (e.g. a 10 cm petri dish with wet Kleenex or KimWipes around the inside edge and sealed with parafilm). Put the needle on a clay mound in the petri dish to prevent sliding.

GOOD LUCK WITH YOUR INJECTIONS!!



Classes of dorsalized phenotypes