

Xenopus Neurite Preparations
(Prepared by Lin Mei with help from Xiao-Hua Xu)

Day 1

Frogs need injection of hCG for egg production. Dosage may vary with the time between injection and egg production. If the dosage is 500 U, the waiting period is around 10 hours; if the dosage is 1000 U, you need to wait around 8 hours. We usually inject at 10 pm on day 1.

hCG (human chorionic gonadotropin), Sigma (cat. # CG-10), 10,000 IU per bottle. Add 10 ml PBS so that the final concentration is 1 IU/microliter. Save as 1 ml aliquots at -20°C (OK for at least one year).

It may be a good idea to take the frog from the frog room to the lab, 4-6 hours before injection to let the frog get used to the lab environment. Use a bucket with some tap water that was sit at room temperature at least for two nights.

Try to find a frog that is active and bright in color. Usually one female frog gives a couple of thousands eggs, of which 500 may be good. When needed, you may use two female frogs.

For injection, hold the frog facing down, head toward the wrist, with the right hind leg between the pointer and the middle finger. Inject 500 microliters of hCG (1 ml syringe, 26G5/8 needle), s.c. along the line between legs and hips on the back.

After injection, let the frog stay in the bucket overnight.

Day 2

Take out testes

Anesthetize the male frog by incubating in ice, 30 min or by destroy the spinal cord.

Add 3 ml of 1x MMR into 60 mm dish.

Surgery: position the frog belly up, take out both testes.

Drop the testes in the 1x MMR in the 60 mm dish.

Cut 1/4 out for next step. The rest can be stored at 10°C for use within a day.

Fertilization

Add 2 ml of 1x Ringer's solution to one corner of 100 mm dish.

Hold the frog the same as Day 1, squeeze out eggs on the dry area of the 100 dish.

Cut 1/4 of one testis in 1x Ringer's solution in the 100 mm dish into tiny pieces with a razor blade.

Mix the eggs with minced testis by adding 0.1x Ringer's solution. Leave at room temperature. Animal poles will turn upright in 20 min after fertilization. Fertilized eggs will develop to the two-cell stage in about 1 hr 20 min.

Preparation of pipettes

Pull the glass pipette (Borosilicate with filament, O.D.: 1.0 mm, I.D.: 0.5 mm, 15 cm length, Cat# BF100-50-15, Sutter Instrument) on pipette puller (Narishige PP-830). First step reading: 55.8; second: 40.3. Observe the tip under Microforge MF-830 (Narishige). Adjust the tip size with forceps to ensure the tip to around 20-30 micrometer.

Preparation of a loading tip. Heat the middle portion of a 200-microliter pipette tip till it melt. Pull the tip end to form a long narrow tubing. Cut the large tip off. Load the modified tip to a 1 ml syringe.

Fill the pipette with RNA/DNA solution (in H₂O). Shake the pipette to remove the bubbles from the tip.

The concentration of DNA/RNA needs to be determined by preliminary experiments. Begin with a low concentration for proteins that are vital for development.

Removal of jelly coat

Incubate fertilized eggs in 1% L-cysteine (C1276, Sigma), pH 8.0 for 3-5 min. Stir with blunt object. Eggs will be rinsed 0.1x Ringer's solution several times. Do not over-digest eggs in L-cysteine, which will affect development.

Note: Jelly coat can also be teased off with fine forceps, save the embryos in 0.1x Ringer's solution at 10-14°C to prevent them from further developing.

Selection of better eggs

Examine eggs in 0.1x Ringer's solution under a dissecting scope. Transfer healthy (clearly divided) eggs with a plastic pipette to a small plate. Eggs can be stored in 0.1x Ringer's solution at 6°C for a few hours.

Injection

Transfer embryos (at two cell stage) to a 60 mm dish with 3% Ficoll-400 in 0.1x Ringer's solution for injection. This is important because eggs may explode after injection if in regular Ringer's solution.

Mount the pipette on a manipulator and connected the Eppendorf FemtoJet system. If the pipette tip contains air bubbles, push the “clean” button to bleed bubbles.

Parameters: Pi (hPa): 60, Ti: 0.1, PC (hPa): 22. The injection volume depends on the tip size, Pi and Ti. The parameters may vary with the size of the pipette. You could also increase the Pi pressure to break a clot in the tip.

Hold an egg using a pair of forceps, lower the pipette into one of the two cells at the animal pole, press the foot paddle once per injection. It is OK if blastmeres pass the two cell stage. In this case, simply inject two or more cells in one blastmere.

It may be better to have mesh plate (spectrum lab, Cat# 145589) at the bottom of the dish to prevent eggs from moving.

It is OK if a fine tip is broken. Reduce Pi pressure to 20 to decrease the injection volume.

Egg explosion or yolk spill indicate that the injection volume may be too big.

After injection

Let injected blastmeres stay in 3% Ficoll/10% Ringer’s solution till stage 17 (8 hours at room temperature). Transfer embryos to 0.1x Ringer’s solution, room temperature overnight. Leave the embryos develop to stages 20-22 at room temperature for neuromuscular coculture, which takes about 20-22 hours after fertilization.

Day 2 (also)

Coat the coverslips (22 x 22 mm, Fisher, cat# 12-545-83) using the following protocol (modified from James Zheng’s lab).

Note: coating may change the response of neurites to certain guidance cues. However, neurites grow better on coated coverslips.

Solutions

1. CMF-PBS

NaCl:	8 g
KCl:	0.30 g
Glucose:	2.0 g
NaH ₂ PO ₄ ·H ₂ O:	0.25 g
NaHCO ₃ :	2 ml of 2% NaHCO ₃

pH 7.4, sterilize by filtration

2. Borate buffer

- (1) Add 2.38 g boric acid (Sigma B0252) and 1.27g borax (B9876) in 500 ml dH₂O;
- (2) Stir for 15 min or until all powder is dissolved;
- (3) Sterilize the buffer by filtering through 0.2 µm filter;
- (4) Label and store at 4°C (Shelf-life: 6 month).

3. Poly-d-lysine (Sigma P1024)

500 µg/ml in borate buffer (Do not filter).

4. Laminin (Sigma L-2020).

- (1) Dissolve 1 mg laminin in 1 ml of CMF-PBS;
- (2) Aliquot into small vials at 20 µl/vial;
- (3) Store at -80°C.

Procedures

1. Take out necessary aliquots from -80°C to thaw them;
2. Clean No. 1 glass coverslips (VWR 40x22 No. 1) with 75% ethanol, rinse in 95% ethanol, flame dry, and put one coverslip in each culture dish;
3. Coat the coverslips with poly-d-lysine for 1hr at room temp, then aspirate the PDL solution from edge. Rinse the coverslips three times w dH₂O.
4. Add 980 µl CMF-PBS to laminin aliquot (20 µg/20ul/tube) to make the final concentration of laminin of 20ug/ml;
5. Add about 250 µl of laminin solution (20 µg/ml) to each coverslip and spread the solution over the coverslip, incubate at 37°C for 2-3 hr and air dry at the room temperature.
6. Close the lid of the culture dish and seal it with parafilm and store overnight at 4°C for next morning cell plating.

Day 3

Dissection of neurotubes

Examine embryos under scope to select injected ones (expressing GFP).

Transfer embryos (stage 20-22) to a 60 mm dish containing 0.1x Ringer's solution.

Take off the remaining jelly coat using needles (mounted on syringe for better handle).

Wash embryos with 1x MMR three times. Transfer embryos each wash to a new dish (35 mm) using a transfer pipette.

Soak the forceps in 70% EtOH.

Hold embryos with forceps and dissect with fine scissors and dissociate neurotubes and associated myotome from embryos.

Be careful that forceps and scissors be kept sterile.

Be careful not to break up the belly of the embryo which may release yolk, which may be adhesive to neurites.

Culture of cells

Transfer neurotubes in CMF (calcium free solution) in 35 mm dish.

Incubate at room temperature for 20-30 min, when neurotubes become loosely dissociated.

While incubation, pull Pasture pipettes for plating cells to have a fine thin tips.

Lay out 35 mm dishes, each containing a coverslip. Roughly three coverslips per two neurotubes.

To each dish, add 1.2 ml of culture medium (50% L15, 49% 1xRinger, 1% FBS). The medium does not have to cover the dish as long as it covers the coverslip.

Suck up neurotubes with a Pasture pipette with a rubble bubble. Lay cells across the coverslips 7-8 times.

Incubate cells at room temperature for 14 hours.

Recipes:

Ringer solution(mM):NaCL 115; CaCL₂ 2; KCL 2.5; Hepes 10. pH 7.4

MMR(mM): NaCL 140; KCL 2.5;MgCL₂ 1; CaCL₂ 1; Hepes 10. pH 7.4

CMF(mM): NaCL 140; KCL 2.5;EDTA 2; Hepes 10. pH 7.4

Culture medium: 50% L-15 (Gibico), 49% Ringer's solution, 1% FBS

Day 4

Pipette preparation and neuron selection

Pull micropipettes (Borosilicate glass, O.D.: 1.0 mm, I.D.: 0.50 mm, 10 cm length, Cat#: B100-50-10, Sutter Instrument) with Narishige Puller (Model PP-830). The tip should be around 1 µm. (Parameters: step 1: 63.5°C; Step 2: 41°C, which may vary).

Load a pipette with a guidance cue (such as netrin) as in Day 2. Use fingers to tap the pipette to make sure that bubbles are cleared from the tip.

Transfer coverslips with coculture to 35-mm cover (inverted) filled with 1x MMR.

Examine neurons under scope (40X). Select a neuron and adjust the position so that a neurite points an angle of 45° with respect to the initial direction of neurite extension (indicated by the last 10 µm segment of the neurite). The growth cone should be centered at the cross.

Pressure system

Turn on N2, with outlet pressure 10-20

Watch the pressure indicator on Pressure System II to be around 15 psi.

Turn on Master-8 to generate pulse and Pressure System II. Master-8 is set at 2 Hz, 20 ms duration.

Push the “select” button to turn on the output (either right or left side). Start with 5-6 ms, which may increase to 20 ms, depending on the tip size.

Mount the pipette on a manipulator connected to the pressure system. Lower the tip closer to the coverslip (the closer the better). Too far away from coverslips may generate wave pulse that may disturb neurons/growth. It is important that the micropipette is placed 100 µm away from the center of the growth cone.

Recording

Open Scion Image.

Go to Special to select Start Capture.

Go to Special again for Average Frame. In this window, select Integrate and Video Rate. Number of Frames should be at 8 or 10. Leave other parameters empty.

Save files as Bitmap files.

Turn off the scope light to decrease damage to neurons.

Watch periodically to ensure pulse is working. If not, increase the pressure to blow off the clot.

Room light may be dimmed for better image or to decrease light toxicity.

Data analysis

Measure turning angle 60 min later. Compare time 0 and time 60 (min) images. There may be a few turns in between, which can be ignored. Neurite outgrowth in between is an important indicator of condition. The growth rate should be larger than 5 $\mu\text{m/hr}$. Turnings of neurites growing at a smaller rate should be discarded.

The turning angle was defined as the angle between the original direction of neurite extension and a straight line connecting the positions of the growth cone at the beginning and the end of the 1-hour period.

3.5 digest in CMF for 20-30 min (remove skin during this period of time)

3.6 plate cells on coverslips with a prepulled pipette

3.7 leave the plated cells to differentiate in room temperature

5.6 transfer embryos to 0.1X Ringer's solution till 20 stage