Fertilization of *Xenopus* Oocytes and Injection of *Xenopus* Embryos

Injection of female frogs with hormone: Do the injections about 20 h before you want to harvest the eggs; Depending on the size, inject female frogs with 500-900 I.U. of human chorionic gonadotropin (hCG; Sigma CG-10; dissolve 10000 I.U. in 10 ml of sterile water; snap freeze and long-term store at -80°C; once thawed, store at 4°C for up to 10 days) into the dorsal lymph sac using a 27-gauge needle; Keep the frogs at 16-18°C;

Squeeze eggs from a frog into a 5.3 cm petri dish (glass or plastic) filled with 1x MMR; Hold the frog by grabbing it from above with your forefinger between the legs and by covering the head/eyes between your palm and your pinky finger; Most frogs will squeeze out their eggs all by themselves if you just hold them and let them try to get out of your grip by struggling; Sometimes it might be necessary to gently exert some pressure by moving your fingers laterally and ventrally over the abdomen; Some frogs secrete copious quantities of a milky exudates from their skins when squeezed; If any of this secretion drips into a petri dish it will induce lysis of a fraction of the eggs, and the contents of the dish should be discarded; Any batch of eggs that shows more than 5% spontaneous lysis, extensive pigment mottling or variegation, or more than 10% spontaneous activation should also be discarded; Do fertilization within 15 minutes after squeezing the eggs;

Fertilization: Remove most of 1x MMR; Using two tweezers, take out a testis by grapping on to the attached partial fat body and dump it into the petri-dish; Using the tweezers like knives, macerate a little piece of the testis beginning from its tip (sacrifice the more, the older the testis is); Vigorously mix the cloud of sperm that you have produced with the eggs by using the tweezers to pull the testis back and forth through the dish; Repeat this 2-4 times (the more often, the older the testis is); Fill dish all the way with 0.1x MMR; This treatment ensures that the subsequent activation is complete; Incubate for about 20 minutes at 18-20°C until all embryos are activated; Activation causes a contraction of the pigmented region of the cortex, and the white spot at the center of the pigmented region becomes less distinct; All the eggs will eventually turn so that the pigmented region is facing up; During this time:

Dejellying of embryos: Prepare dejellying solution: 2% cysteine in H_2O; Adjust pH to 7.8 with 5 M NaOH; do not store for more than 90 minutes before use; Remove as much of the 0.1x MMR as possible and replace with dejellying solution; Incubate until embryos pack tightly together (takes about 5 minutes); Do not swirl unnecessarily; When the jelly coat is gone from all the embryos: Wash 6 times with 0.1x MMR; Pick “bad” embryos (lysed or strangely pigmented) with a Pasteur pipette and discard; Transfer embryos into fresh petri dishes filled with 0.1x MMR by using a Pasteur pipette whose tip has been cut and broken off to give an orifice about 4 mm in diameter and then fire-polished; Transfer as little buffer as possible at this step to get rid off all traces of cysteine; Incubate aliquots of embryos at different temperatures (e.g. 20, 18, 16 and 14°C) because this will later allow you to inject the same stage embryos (e.g. two-cell) over a longer period of time; At 18°C it will take about 2 hours from the time of fertilization until the embryos cleave for the first time; During this time:

Prepare the needle for injection: Carefully put a needle under the stereo-microscope (WILD Heerbrugg, M5A, 10x eyepiece, one of which has a graduation); At 50x

magnification, break off the tip of the needle using fine tweezers; The outer diameter of the needle should be around 3/4th of the distance between two pitch lines of the graduation, which corresponds to 15 μm (at 50x magnification 1 mm corresponds to 50 pitch lines); The inner diameter should be about half the distance between two pitch lines; Don't break off too much; Instead, brake off the needle, measure the diameter of the tip, and then break off more if needed; Try to break the needle so that you will get a pointy end, which is indicated by its bright reflection of light; Put the needle with its blunt, wide end into the tubing of the PLI-100 injector and screw the needle gently into the holder of the micromanipulator; When injecting RNA, wear gloves and work RNAse-free;

Open on the "compressed air" outlet that connects to the PLI-100 injector; Put the "pressure meter source"-knob in the P_{clear} position; The reading should be between 60 and 90 psi and stay fairly constant; Put the "pressure meter source"-knob in the P_{inject} position; Adjust the reading to 10 psi as a starting point (it should be between 5 and 15 psi); Set the injection time to 400 msec (it should be between 300 and 500 msec);

Calibrate the needle: Before putting a petri dish under the microscope (or removing one) always remember to retract (and thereby higher) the needle far enough so that you will not brake off the tip! Through the tip, suck up H_2O from a petri dish to fill the needle; To do this, press the "fill" button or step onto the corresponding foot pedal; Put a petri dish filled with mineral oil (Sigma 400-5, heavy white oil, viscosity (100°F): 340-360 saybolt universal seconds, specific gravity (77°F): 0.875-0.885, reuse) under the microscope and move the tip of the needle into the oil while watching it at 50x magnification; Inject H_2O into the oil by stepping onto the "inject" foot pedal; Immediately after injection, pull back the needle and out of the oil using the micromanipulator; Using the graduation of the eyepiece, estimate the diameter of the H_2O-droplet, it should be 12-13 pitch lines (50x magnification); This size of drop correspond to an injection volume of about 10 nl; If the droplet is too small or too big increase or decrease, respectively, the injection time (however, keep it between 300 and 500 msec); Alternatively/additionally you can increase or decrease, respectively, the injection pressure (however, keep it between 5 and 15 psi); If you need too high pressure and/or too long time to get the right size droplet, break off the tip a little more; Wash needle free of oil by submersion into water and by going through two cycles of filling and ejecting; For rapid ejection: Press the "clear" button;

Loading the needle with sample: Put your sample as a small droplet (typically 3 μl) down onto a piece of parafilm in a petri dish; Set the magnification to 12x; Focus onto the top of the drop and center the drop in the middle of your visual field by moving the dish; By using the screws of the micromanipulator, position the needle so that you can pinch the drop's surface with the tip of the needle by just using the screw that will move the needle forward; Submerse the tip of the needle just below the surface of the drop and fill the needle with your sample; RNA is typically used at a concentration of 0.2 to 200 ng/μl (in H_2O), which corresponds to roughly 2 pg to 2 ng per injection; When injecting DNA (plasmid-DNA in H_2O) the rule-of-thumb is to use 10 fold less than RNA and at maximum 200 pg; With DNA expression does not start until MBT and expression levels vary greatly between cells depending on how DNA was partitioned; When injecting protein: Use as much as possible but do not inject more than 20 nl per each cell of 2-cell embryos

Adjust the balance pressure: To this end, submerse the needle tip into 0.1x MMR / 5% Ficoll (Sigma F-4375, MW about 400000, type 400; prepare the solution in advance; stir for a couple of hours at room temperature to get the Ficoll into solution; store at 4°C) and focus onto its tip; Put the "pressure meter source"-knob in the $P_{balance}$ position; Adjust the balance pressure to the lowest value where you just see cords of water being slowly ejected into the Ficoll-containing solution; This should be the case at – 0.5 to 0.5 psi; The balance pressure avoids sucking of liquid into the needle due to capillary action;

Actual Injection: 12x magnification; Transfer embryos (typically 2-cell stage but an injection volume of 10 nl is okay for up to 8-cell stage) in one corner of the petri dish filled with 0.1x MMR / 5% Ficoll; Using tweezers (which you keep closed at all times and use only as a tool to push embryos around) that you hold in your left hand, move a nicely looking embryo (much of the quality control takes place at this stage) into the center of the dish, where your needle will appear upon moving it forward; Focus onto the embryo surface; Move the needle forward and push it into the darkly pigmented region at the upper third of the embryo; Hold the embryo in place by positioning the tweezers on the other side; Immediately retract the needle a little bit to ensure injection close to the cell surface; Press the "inject" foot petal; You should see a whitening of the cell surface at the side of injection due to replacement of pigment by the injected sample; Immediately after injection, pull the needle out of the embryo; Move the injected embryo to an empty area of the dish; Repeat many times; Every now and then, transfer injected embryos into new petri dish with 0.1x MMR (avoid transfer of to much Ficoll) and add new, uninjected embryos to your dish with 0.1x MMR / 5% Ficoll; Remember: Always keep an embryo as "buffer" between the forceps and the needle tip; Never touch the needle with the tweezers; After injection: Incubate embryos at 18°C;

Fix the embryos in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde); Kill the embryos with benzocaine solution (see above) prior to fixation if they are already in tadpole stage