

(last modified: March 12, 1999)

Transgenesis in Xenopus

Enrique Amaya

Wellcome/CRC Institute, Tennis Court Road, Cambridge, CB2 1QR, UK

1. Introduction

We recently developed a very efficient method for generating transgenic frog embryos (Kroll and Amaya, 1996). Briefly the protocol involves the following steps: 1. Sperm nuclei are incubated with linearised plasmid DNA. 2. After a short incubation, a high-speed interphase egg extract and a small amount of the restriction enzyme are added to the sperm nuclei and plasmid mixture. The extract partially decondenses the sperm chromatin but does not promote replication and the restriction enzyme stimulate recombination and integration by creating double stranded breaks in the sperm chromatin. 3. After the plasmid-treated nuclei are incubated for a brief period in the interphase extract, the mixture is diluted and the nuclei are transplanted into unfertilised eggs, resulting in the production of transgenic embryos. This technique permits large scale transgenesis in *Xenopus* embryos. Unlike embryos injected with plasmids, the transgenic embryos show correct spatial and temporal regulation of integrated promoter constructs. One of the great advantages of this system over transgenesis in mice or zebrafish is that the transgene is integrated into the male genome prior to fertilisation, therefore the resulting embryos are not chimeric and breeding of animals is not required. In other words, transgenic embryos can be generated one day and analysed the next. This new technology can be used to 1) misexpress genes during development with much better spatial and temporal control, 2) label specific structures *in vivo*, such as the musculature or nervous system, 3) study the regulation of promoters of genes from amphibia, zebrafish and mouse, 4) study the molecular basis of organogenesis and 5) generate mutations in genes through gene trap approaches.

In this chapter I would like to describe in detail the protocol for generating transgenic frog embryos. It includes a section on how to prepare high speed interphase extracts from eggs, a section on how to prepare sperm nuclei, and a section on how to integrate DNA into isolated sperm nuclei and transplant the

nuclei into eggs, thus generating transgenic embryos. For up to date information on the procedure, see the Amaya Lab website (<http://www.welc.cam.ac.uk/~ea3/>).

2. High Speed Extract Preparation

A. Stock Solutions

10X Marc's Modified Ringer's (MMR) (1 M NaCl; 20 mM KCl; 10 mM MgCl₂; 20 mM CaCl₂; 50 mM HEPES, pH 7.5) (Sterilise by autoclaving and store at room temperature.

20X Extract Buffer (XB) salts (2M KCl; 20 mM MgCl₂; 2 mM CaCl₂; filter sterilise and store at 4°C

1.5 M Sucrose (filter sterilise and store 8.33 ml aliquots at -20°C)

1 M HEPES (titrate with KOH so that when diluted to 10 mM the pH is 7.7, should require that the pH of the 1M stock is around 9.5; filter sterilise and store 0.75ml aliquots at -20°C) Note: Dilution drastically changes the pH of HEPES.

1M CaCl₂ (filter sterilise and store at 4°C)

1M MgCl₂ (filter sterilise and store at room temperature)

200mM EGTA (adjust pH to 7.7, sterilise by autoclaving and store at room temp.)

Versilube F-50. Made by General Electric. Can be purchased from Andpak-EMA [1560 Dobbin Drive, San Jose, CA 95133; Tel. (408)-272-8007].

Energy Mix (150 mM creatine phosphate (Boeringer Mannheim Biochemicals 127 574; 20 mM ATP (Boeringer Mannheim Biochemicals 519 979; 20 mM MgCl₂; made up in H₂O; store 0.1 ml aliquots at -20°C)

100 U/ml Pregnant mare serum gonadotropin (PMSG; 367222; Calbiochem; made up in sterile H₂O; store 10 ml aliquots at -20°C.)

1,000 U/ml Human chorionic gonadotropin (Sigma CG-10; made up in sterile H₂O; stored at 4°C.

10 mg/ml leupeptin (Boeringer Mannheim 1017 101; in DMSO; store 40 μ l aliquots at -20°C.)

10 mg/ml chymostatin (Boeringer Mannheim 1 004 638) and pepstatin (Boeringer Mannheim 600 160); in DMSO; store 40 μ l aliquots at -20°C.)

B. Solutions for High Speed Extract Preparation.

On the day of the extract preparation, make up the following:

4000 ml 1X MMR

3600 ml H₂O

400 ml 10X MMR

1000 ml 2% (w/v) cysteine in 1X XB salts

900 ml H₂O

50 ml 20 XB salts

20g cysteine (Sigma C-7755)

titrate to pH 7.8 with 5N NaOH. Make up within 1 hour of use.

500 ml Extract Buffer (XB)

450 ml H₂O

25 ml 20X XB salts

16.7 ml 1.5M sucrose

5 ml 1M HEPES

Final concentration

100mM KCl; 1mM MgCl₂; 0.1 mM CaCl₂

50mM Sucrose

10mM HEPES, pH 7.7

75 ml CSF-XB

	<u>Final concentration</u>
66 ml H ₂ O	
3.75 ml 20X XB salts	100mM KCl; 2mM MgCl ₂ ; 0.1 mM CaCl ₂
75 µl of 1M MgCl ₂	
2.5 ml 1.5M sucrose	50mM Sucrose
0.75 ml 1M HEPES	10mM HEPES, pH 7.7
1.88 ml 200mM EGTA, pH 7.7	5mM EGTA
75 µl 10 mg/ml chymostatin/pepstatin mix	10 µg/ml
75 µl 10mg/ml leupeptin	10 µg/ml

C. High Speed Extract Preparation

The high speed extract preparation is largely based on Murray (1991). Briefly, a crude cytostatic factor (CSF) arrested egg extract (cytoplasm arrested in meiotic metaphase) is prepared. Calcium is added to drive the extract into interphase and a high speed spin is performed to obtain a purer cytoplasmic fraction.

Cytochalasin is omitted because it interferes with normal development.

Although a crude extract can be used for making transgenic embryos, high speed extracts have several advantages over crude extracts. 1) High speed extracts promote decondensation of sperm nuclei but do not promote DNA replication; 2) high speed extracts cannot progress through the cell cycle; 3) high speed extract can be stored frozen (at -80°C) for many months before use; and 4) sperm nuclei are more easily mixed in high speed extracts which do not contain actin polymers.

1) Prime 8-12 female adult *X. laevis* about 48 hours prior to HCG injection by injecting 50U of pregnant mare serum gonadotropin (PMSG) into the dorsal lymph sac. Maintain at room temperature. The evening before the extract preparation, inject each frog with 500U HCG and place two frogs per container

into 2 liters 1X MMR. Since one frog with lysing or activating eggs can compromise the whole extract preparation, we prefer to separate the frogs into pairs for the ovulation. The frogs are then placed at 18-19°C overnight (12-14 hours). The next morning the egg quality from each container is screened before mixing all the eggs and starting the extract preparation. All the eggs released from a frog which lays mottled, lysing or dying eggs are left out of the extract preparation.

2) All solutions should be prepared before beginning the extract preparation since the procedure should be carried through all steps promptly once it is initiated; optimally, the high speed spin should begin within 45-60 minutes of dejellying the eggs.

3) Overnight the frogs should have laid eggs into the 2 liters of 1X MMR. The high salt in 1X MMR should keep the eggs unactivated and healthy. Manually expel any remaining eggs from each frog into the 2 liter of 1X MMR. Remove the frogs and transfer the eggs from each container into separate 500ml beakers containing around 50ml of 1X MMR. If the eggs look unhealthy, eliminate the eggs from the prep.

3) Remove as much MMR as possible from the eggs. Dejelly eggs in 2% cysteine in XB salts (no HEPES/sucrose) at pH 7.8. Add a small amount at a time, swirl eggs, and partially replace with fresh cysteine several times during dejellying. Dejellying should be performed separately for different batches of eggs and batches which show breakage or egg activation during dejellying should be discarded.

4) Once the eggs from all the batches have been dejellied, the remaining good eggs are pooled and washed thoroughly in XB (with HEPES/sucrose). We use about 125 mls for each wash and do 4 washes.

5) Wash eggs in CSF-XB with protease inhibitors. We do two 35 ml washes.

6) Using a wide-bore pasteur pipette, transfer eggs into Beckman ultraclear tubes. For these volumes, we typically use 14X95 mm tubes (Catalog #: 344060; Beckman, Fullerton, CA 344057). If multiple tubes will be used, try to transfer an equal volume of eggs per tube. Remove as much CSF-XB as possible and replace with about 1 ml of Versilube F-50.

7) Spin in a clinical centrifuge at room temperature for about 60 seconds at 1000 rpm (150 g) and then 30 seconds at 2000 rpm (600 g). Eggs should be packed after this spin but unbroken. Versilube should replace the CSF-XB between the eggs and an inverted meniscus between the Versilube and displaced CSF-XB should be clearly visible. Remove the excess CSF-XB and Versilube and then balance the tubes.

8) Spin the tubes in rubber adapters for 10 minutes at 10,000 rpm at 2°C in Sorvall HB-4 or similar swinging bucket rotor to crush the eggs. The eggs should be separated into three layers: lipid (top), cytoplasm (center), and yolk (bottom). Collect the cytoplasmic layer from each tube with an 18 gauge needle by inserting the needle at the base of the cytoplasmic layer and withdrawing slowly. Transfer cytoplasm to a fresh Beckman tube on ice. If large volumes of darkly pigmented eggs are used, the cytoplasmic layer may be greyish rather than golden at this step. After a second spin to clarify this extract, it should be golden.

- 9) Estimate the volume of extracts and add the appropriate amount of protease inhibitors to the isolated cytoplasm (do not add cytochalasin); recentrifuge the cytoplasm in Beckman tubes for an additional 10 minutes at 10,000 rpm to clarify, again using a swinging bucket rotor. Collect the clarified cytoplasm as before. Expect to get about 0.75-1 ml cytoplasm per batch of eggs collected from one frog.
- 10) Add 1/20th volume of the ATP-regenerating system (Energy Mix). Transfer the clarified cytoplasm into TL100 tubes thick wall polycarbonate tubes (Beckman 349622). Tubes hold about 4 mls each and should be at least half full.
- 11) Add CaCl_2 to each tube to a final concentration of 0.4mM; this inactivates CSF and pushes the extract into interphase. Incubate at room temperature for 15 minutes then balance for the high speed spin.
- 12) Spin tubes in a Beckman tabletop TL-100 ultracentrifuge in a TL100 rotor (fixed angle) at 70,000 rpm for 1.5 hours at 4°C.
- 13) The cytoplasm will fractionate into four layers, top to bottom: lipid, cytosol, membranes/mitochondria, and glycogen/ribosomes. Remove the cytosolic layer from each tube (about 1 ml if 2-3 ml was loaded into the tube) by inserting a syringe into the top of the tube through the lipid layer. Transfer this fraction to fresh TL-100 tubes and spin again at 70,000 for 20 min. at 4°C.
- 14) Aliquot the high speed cytosol supernatant into 15 μl aliquots in 0.5ml eppendorf tubes. Quick freeze aliquots in liquid nitrogen and store at -80°C until use. We typically obtain 2-3 ml of high speed cytosol from preparations of this scale. Sperm nuclei should be incubated in an aliquot of extract and stained with Hoechst to determine whether extract is active in decondensation. Active

interphase extract should visibly swell sperm nuclei (thicken and lengthen) within 10-15 minutes of addition to extract at room temperature.

2. Sperm Nuclei Preparation

A. Stock Solutions

10X Marc's Modified Ringers (MMR) (1 M NaCl; 20 mM KCl; 10 mM MgCl₂; 20 mM CaCl₂; 50 mM HEPES, pH 7.5) (Sterilise by autoclaving and store at room temperature.

1.5 M Sucrose (filter sterilise and store 8.33 ml aliquots at -20°C)

1 M HEPES (titrate with KOH so that when diluted to 15 mM the pH is 7.7, should require about 5.5 mls of 10 N KOH for 100 mls; filter sterilise and store 0.75 ml aliquots at -20°C)

Note: Dilution drastically changes the pH of HEPES, making it impossible to pH the stock directly.

10 mM spermidine trihydrochloride (filter sterilise and store 2.5 ml aliquots at -20°C)

10 mM spermine tetrahydrochloride (filter sterilise and store 1 ml aliquots at -20°C)

100 mM Dithiothreitol (filter sterilise and store 0.5 ml aliquots at -20°C)

500 mM EDTA pH 8 (stored at room temperature)

10% Bovine Serum Albumin (BSA; fraction V), (in H₂O; store 3.5 ml aliquots at -20°C)

10 mg/ml leupeptin (Boeringer Mannheim 1017 101; in DMSO; store 40 µl aliquots at -20°C.)

0.3 M phenylmethylsulfonyl fluoride (PMSF) (in EtOH, store at -20°C)

100% glycerol (sterilise by autoclaving and store at room temperature)

10 mg/ml Hoechst No. 33342 (in dH₂O; store in a light-tight vessel at -20°C)

100 μ l of 10 mg/ml L- -lysophosphatidylcholine (Lysolecithin) (Sigma L-4129 or CalBiochem 440154); dissolve in H₂O at room temperature **immediately before use**. Store solid stock desiccated at -20°C. Discard the stock powder if it becomes sticky.

10 ml of 1X NPB + 3% BSA w/ protease inhibitors

2 ml H₂O

5 ml 2X NPB

3 ml 10 % BSA

10 μ l 10mg/ml leupeptin (added immediately before use)

10 μ l 0.3 M PMSF (added immediately before use)

store on ice

5 ml of 1X NPB + 0.3% BSA

2.35 ml H₂O

2.5 ml 2X NPB

0.15 ml 10 % BSA

store on ice

0.5 ml 1X NPB + 30% glycerol + 0.3% BSA (Sperm Storage Buffer)

85 μ l H₂O

250 μ l 2X NPB

15 μ l 10 % BSA

150 μ l 100 % glycerol

store on ice

C Sperm Nuclei Preparation (largely based on Murray, 1991).

1) Anaesthetise a male by immersion in 0.1% aminobenzoic acid ethyl ester (Tricaine; MS222) (Sigma A-5040) with 0.1% sodium bicarbonate for at least 20 minutes and then pith it. Cut through the ventral body wall and musculature and lift the yellow fat bodies to isolate the two testes which are attached to the base of the fat bodies one on each side of the midline. Remove the testes with dissecting scissors and place them in a 35mm tissue culture dish containing cold 1X MMR. We routinely isolate sperm nuclei from one testis and use the other for *in vitro* fertilisations. Inspect the two testes and isolate the one with less blood contamination for the nuclear prep. (We store the other testis in 1X MMR, 10% fetal calf serum, and gentamycin in a vial at 4 °C and use it for *in vitro* fertilisations for up to a week.) If a large prep is required, testes from two to four males can be used. The final resuspension volume should be increased accordingly.

2) Rinse the testis in three changes of cold 1X MMR.

3) Using fine forceps, remove any remaining fat body and excess blood. Do not try to remove the blood vessels. Rather, puncture holes in the largest vessels and gently push the blood out. Take care not to puncture the testis as this releases sperm.

2) Place the cleaned testis in another 35mm tissue culture dish with 5 ml of cold 1X NPB + protease inhibitors for 2 to 5 minutes.

3) Transfer the testis to a dry 35mm tissue culture dish, and macerate the tissue well (until clumps are no longer visible to the naked eye) with a pair of clean forceps.

- 3) Resuspend the macerated testes in 2 mls of cold 1X NPB + protease inhibitors by pipetting the mixture up and down through a sterile, disposable 5 ml pipette.

- 4) Squirt the sperm suspension through two-four thicknesses of cheesecloth placed into a funnel and collect the solution into a 14 ml sterile culture tube (Falcon 2059; 17 x 100 mm). Rinse the dish with an additional 3 ml of cold 1XNPB + protease inhibitors and add to the cheesecloth. After adding 5 ml more (10 mls total) of cold 1XNPB + protease inhibitors use a gloved hand to fold the cheesecloth and squeeze any remaining liquid through the funnel into the 14 ml tube. We usually end up with 9 ml of sperm suspension in the tube.

- 5) Centrifuge the sperm suspension at 3,000 rpm for 10 min. at 4°C (we use a Sorvall HB-4 or similar swinging bucket rotor fitted with the appropriate adapters). The sperm pellet should be white, fairly compact. Usually we have some blood contamination which can be seen in the center of the pellet. During the spin, allow 1 ml of 1XNPB + protease inhibitors to equilibrate to room temperature.

- 6) Decant the supernatant and resuspend the sperm pellet in 9 ml of cold 1XNPB + protease inhibitors and repellet by centrifugation at 3,000 rpm, 10 min., 4°C. During this spin dissolve 1 mg of L- -lysophosphatidylcholine (Lysolecithin) (Sigma L-4129) in 100 ul (10mg/ml) of H₂O at room temperature. Lysolecithin will not remain in solution below room temperature.

- 7) Decant the supernatant and resuspend the sperm pellet in the 1 ml of 1XNPB + protease inhibitors that has equilibrate at room temperature and add 50µl of 10mg/ml lysolecithin. Mix gently and incubate for 5 minutes at room temperature.

8) Add 10 ml cold 1XNPB +3% BSA + protease inhibitors to the suspension and centrifuge at 3,000 rpm, 10 min., 4°C. At the end of this spin the pellet should now be wider and more loose than before. In addition the pellet should no longer have redness. The looseness and the loss of haemaglobin mean that the pellet now contains nuclei rather than intact cells.

9) Decant the supernatant and resuspend the pellet in 5 ml cold 1XNPB + 0.3% BSA (no protease inhibitors), mix gently by pipetting up and down, and centrifuge at 3,000 rpm, 10 min., 4°C.

10) Decant the supernatant and resuspend the pellet in 250µl of 1XNPB + 30% (w/v) glycerol + 0.3% BSA (Sperm Storage Buffer) and transfer suspension into a 1.5 ml eppendorf tube. Store at 4°C and use for transgenesis for up to 48 hours.

11) Cut the tip of a yellow tip with a razor blade and mix the sperm nuclei suspension by pipetting up and down. Remove 2 µl and dilute into 200 ul of sperm dilution buffer (i.e. 1:100 dilution). Add 2 µl of a 1:100 diluted Hoechst stock and transfer the diluted sperm nuclei to a hemacytometer for counting. Visualise the sperm nuclei under a fluorescence microscope using a DAPI/Hoechst filter set. For a 1:100 dilution of our sperm nuclei stock, we typically obtain counts of 125-200 ($\times 10^4$ nuclei/ml). At this concentration, the undiluted stock contains 125-200 nuclei/nl. If your sperm stock is substantially less concentrated (i.e.. a count of <100 for a 1:100 dilution), repellet the sperm nuclei at low speed (or allow the nuclei to settle over a few hours) and resuspend in a smaller volume of sperm storage buffer. We store the fresh nuclei overnight at 4°C and after extensive mixing by pipetting up and down with a cut yellow tip, we freeze 40 µl aliquots in liquid nitrogen and store the frozen nuclei at -80°C. One aliquot is thawed for each day of transgenesis.

3. Preparation of DNA, Needles and Equipment

A. Preparation of linearised DNA.

Digest DNA using standard conditions, run the DNA on a 1XTAE agarose gel, isolate the appropriate band and purify using the GeneClean Kit II by Bio101. Elute the DNA in dH₂O at a final concentration of 150-250 µg/µl.

B. Preparation of nuclear transplantation needles.

These needles are unlike standard needles used for DNA and RNA injection in that the diameter of the point is an order of magnitude larger to allow nuclei to pass through intact. 30 µl Drummond micropipets (Fisher, cat. #: 21-170J) are used to make the needles. It is important to pull needles with gentle slopes at the tip. This makes it easier to clip the needles at the desired diameter and also they cause less damage to the eggs during transplantation. We currently use a Flaming/Brown Micropipette Puller Model P-87 (Sutter Instruments Co.) for pulling our needles. The setting depend on the filament used, so should be adjusted so that a gentle slope toward the tip is achieved.

Clip the needle with forceps to produce a bevelled tip of 80-100 µm diameter using the ocular micrometer of a dissecting microscope or a stage micrometer for measurement. It is essential that the tip be this wide or nuclei passing through will be damaged. When clipping tips, it often helps to use forceps with slightly unmatched tips and to pull outward at a 20 or 30 degree angle from the needle as the forceps contacts the needle.

Treat the inside of needles with Sigmacote (Sigma SL-2) to prevent shearing of sperm nuclei flowing through the needle (needles can be coated 10 minutes to several months before use). Attach approximately 1 cm Tygon tubing (R-3603 1/32"; Fisher, cat. #: 14-169-1A) to the end of a plastic pipette tip and

use the pipette to draw up Sigmacote; then attach the other end of the tubing to the injection needle (see below). Depress the pipette plunger to force Sigmacote through the needle until a few drops emerge from the tip then release the pipette plunger to withdraw most of the solution. Rinse needle with at least 200 μ l of water. Make sure to remove all the liquid from the needle as any remaining liquid will block flow of nuclei into the needle.

C) Agarose-coated injection dishes

Pour 2.5% agarose in 0.1X MMR into around 10 60mm petri dishes. Before the agarose solidifies, place small weigh boats on the agarose so that as the agarose solidifies, a square depression in the agarose remains. The depression will accommodate ~500 eggs. Wrap the dishes in parafilm and store at 4°C until use.

D) Transplantation Apparatus

Most commercial injection apparatuses used for DNA and RNA injections which are based on air pressure are unsuitable for nuclear transplantations, due to the large difference in needle tip size. Flow through the 5 μ m needle tips used for fluid injections is controllable at fairly high pressures. However, with these standard systems it is usually not possible to obtain an extremely low positive pressure and gentle, controlled flow required to deliver an intact nucleus in a small volume (10-15 nl) through a 80-100 μ m needle tip. Therefore, for nuclear transplantation we use a Harvard Apparatus infusion syringe pump (Model 22; Cat.# 55-2222) with two 2.5ml Hamilton Gas Tight Syringes and Tygon R3603 tubing (0.8mm X 0.8mm). This way two people can transplant nuclei at the same time. The oil that we use to fill the system is Mineral Oil (Embryo Tested) from Sigma (Cat. No. M-8410). The big advantage of using an infusion pump is that we can adjust it to any desirable flowrate. We have ours set at 10nl/sec.

4. Transgenesis by sperm nuclear transplantation into unfertilised eggs

1) The night before eggs are needed for transplantations, inject 2 adult female frogs in the dorsal lymph sac with 500-800U Human Chorionic Gonadotropin (HCG) and incubate at 19°C for 10-12 hours.

2) Remove a 1ml aliquot of Sperm Dilution Buffer (SDB) from the freezer and allow it to warm to room temperature.

3) Make up 500ml of 2.5% cysteine in 1X MMR pH 7.8 (with 5N NaOH).

4) Fill agarose coated injection dishes with 0.4X MMR + 6% Ficoll

5) Set up a reaction:

4 µl sperm stock (~4 X 10⁵ nuclei)

2.5 µl linearized plasmid (100 ng/µl)

Incubate 5min at room temperature.

Meanwhile:

Obtain a 15µl aliquot of high speed extract from -80 freezer and allow to thaw to room temperature (only takes a few minutes). Keep aliquot on ice for the day.

Make the following mixture:

18 µl SDB

2 µl extracts

2 µl 100 mM MgCl₂ (add to 5 mM final at all steps to aid enzyme action)

0.5 µl of a ~1:10 dilution of Sal I or Not I to the extracts (depending on the construct).

Mix well.

After 5 minute incubation of DNA with sperm nuclei add the extract:enzyme:MgCl₂ mixture to the sperm nuclei.

Mix the reaction by gentle pipetting (using a clipped yellow tip).

Incubate 15 min. at room temperature.

6) While sperm are swelling in reaction mixture, collect eggs from the frogs and dejelly them in 2.5% cysteine hydrochloride in 1X MMR (pH 8.0 with NaOH). Wash the eggs thoroughly in 1X MMR. Transfer dejellied eggs into agarose coated dishes containing 0.4X MMR with 6% ficoll and gentamycin. We try to fill the depression square with eggs. After about 5 minutes in 0.4X MMR + 6% Ficoll the eggs will pierce easily.

7) After the 10-15 minute incubation with extracts, mix the sperm nuclei gently by pipetting up and down with a cut of yellow tip. Then transfer 5 µl of mixture into 150 µl of SDB that is already at room temperature. At this point do not mix the nuclei. Mixing at this point is likely to shear the nuclei. Allow the sperm nuclei:extract mixture to slowly equilibrate with the SDB over the span of a few minutes.

8) Using a piece of Tygon tubing attached to a yellow tip (as previously described for Sigmacoting needles) draw up the dilute sperm suspension, mix gently by pipetting up and down and then draw up the dilute sperm nuclei and detach the yellow tip from the pipetman (try not to create or leave bubbles in the tygon tubing as these may damage nuclei). Be careful to keep the yellow tip horizontal or the nuclei will dribble out. Now back load a needle by attaching it to the tygon tubing and raising the angle slightly so that the nuclei flow gently into the needle. As long as no liquid is present at the tip of the needle the nuclei should flow easily by simple gravity. Once the needle has backfilled completely with nuclei,

detach the needle and attach it to the tygon tubing filled with mineral oil that is connected to the Harvard Apparatus infusion pump. If two people of injecting load another needle as before. Place the yellow tip with the remaining nuclei aside horizontally in case you need to load another needle.

9) Transplant sperm nuclei into unfertilised eggs.

Start the flow in the infusion pump and begin injecting eggs, keeping the needle inside each egg for approximately one second. Move the needle fairly rapidly from egg to egg, piercing the plasma membrane of each egg with a single, sharp motion then drawing the needle out more slowly. The angle of the needle should be perpendicular to the membrane surface (rather than glancing) to avoid tearing the plasma membrane. If the needle becomes clogged with cytoplasm, bring the tip to the air-liquid interface of the dish. Sometimes the surface tension of the interface removes the cytoplasm plug in the end of the needle. If a needle tip is too narrow, or if it becomes partially clogged with debris during transplantations, the injected nuclei will be damaged during transplantation, resulting in aneuploid or haploid embryos.

You can determine whether your sperm dilution and the flow rate used for injections were appropriate by watching the first cleavage of the transplanted eggs. If few of the eggs received a nucleus, the frequency of cleavage will be low; one fifth to one third of our transplantations typically result in normally cleaving embryos. Eggs that were injected with more than one nucleus will divide at the time of first cleavage abnormally into three or four (or more) cells. Many of these embryos will develop to blastula stages, but most fail during gastrulation; in some, a region of the embryo will fail to cellularize and die. Eggs injected with multiple nuclei which do gastrulate usually do so abnormally; typically, blastopore closure is incomplete resulting in embryos that form two wings of somites and neural tissue on each side of the exposed yolky tissue lying in the

center of the trunk. This type of gastrulation failure is common to stressed or unhealthy embryos (particularly embryos derived from 'soft' eggs).

10) When the embryos have reached the 4-cell stage, gently separate them from uncleaved eggs and transfer them to a separate dish of 0.1X MMR + 6%Ficoll +50µg/ml gentamycin. Do not be fooled by pseudocleavages. Only keep embryos that appear like normal, healthy 4-cell embryos. We commonly culture transplanted embryos in 24-well tissue culture dishes with about 5 embryos per well, since culturing embryos at high density can compromise their health. It is also important to remove dying embryos promptly since they also can compromise the health of their siblings. When embryos are around stage 12, media is replaced with 0.1X MMR + 50µg/ml gentamycin without Ficoll. Because of the large needle tip used for transplantations, embryos often develop large blebs at the site of injection. These blebs occur when cells are forced out of the hole left in the vitelline membrane at the injection site but they generally do not affect development. The blebs usually fall at the neurula or tailbud stages, but they can be removed manually once the embryos have reached the late blastula stage.

5) References

Kroll, K.K. and Amaya, E. (1996) Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* 122:3173-3183.

Murray, A.W. (1991). Cell cycle extracts. In *Methods in Cell Biology*, (B. K. Kay, and H. B. Peng), ed., Vol. 36, pp. 581-605. San Diego: Academic Press, Inc.