

EMBRYO MICROINJECTION AND BLASTULA STAGE CELL TRANSPLANTATION

Materials:

- 10% Hanks (>1 L) or embryo medium (preferably), verified pH 7.2–7.6
- system water (> 1L)
- aliquot of 20 mg/mL pronase (Sigma P8811)
- 1x 50 mL screw cap Falcon tube
- 250 mL glass beakers filled to ~200 ml with 10% Hanks
- 100 mm petri dishes lined with a thin layer of 1% agar (made in 10% Hanks) containing ~30 mL 10% Hanks or embryo medium; or, preferably, 100 mm reusable glass petri dishes (no agar lining needed)
- 100 mm bevelled injection dishes made with 1% agar (made in 10% Hanks) containing ~20 mL 10% Hanks or embryo medium
- penicillin/streptomycin solution (5000IU/mL, 5000g/mL)
- fire-polished glass pasteur pipettes and pipette pump
- depression slide
- mineral oil (white, heavy; Sigma 330760)
- ocular and stage micrometers
- 60 mm petri dishes lined with 1% agar containing embryo medium (for transplants)
- embryo medium (for transplants)
- ice bucket for holding mRNA, etc.
- injection needles, filament (World Precision Instruments 1.0 mm x 4 in w/fil #TW100F-4)
- injection needles, no filament (Harvard Apparatus 1.0 mm OD x 0.58 mm ID #GC100-10)

Preparing injection solutions

For injecting DNA using the Tol2 transposase system:

- Miniprep DNA using the Parichy lab alkaline-lysis phenol-chloroform protocol and resuspend the pellet in TE
- Determine plasmid concentration using the nanodrop spectrophotometer: there should be 1000–3000 ng/μL of plasmid, if less, something has gone wrong with the prep.
- Verify plasmid identify and DNA quality by diagnostic restriction digest.
- Remove ~half of the alkaline lysis miniprep to prepare for injection. Store the remainder appropriately (e.g., in the long-term clones storage boxes at –80 °C)
- Further purify the DNA to be injected using a Qiagen PCR Purification kit (do not use off-brand columns) and elute using 40 μL of HPLC grade water (do *not* use TE, Tris, EB elution buffer, etc.). Handle the materials so as to keep the plasmid RNase free throughout the process. Determine the concentration with the nanodrop spectrophotometer (should be 80–300 ng/μL).
- Dilute a sample of the injectable plasmid to 50 ng/μL and mark diluted and concentrated stocks with “Q” or “inj” to indicate they contain DNA of injectable quality. Store injection DNA stocks long-term at -20 °C.
- For injecting, you will want to deliver enough DNA so that 30–50% of the embryos will exhibit defects associated with DNA toxicity by 24 h (e.g., central nervous system necrosis). This quantity of DNA needs to be determined empirically but 12.5 pg per embryo is a good starting point. You will typically want to inject a ~2 nl bolus of solution to ensure that DNA is spread widely in the cytoplasm, so you will need to dilute the injection solution to a final DNA concentration of 12.5 pg / 2 nl = 6.25 pg/nl = 6.25 ng/μL. The injection solution should contain an equal amount of *Tol2* mRNA. Therefore, if you are starting with 50 ng/μL plasmid DNA and 50 ng/μL *Tol2* transposase mRNA (stored in single session aliquots

at -80 °C), and wish to make 8 μL of injection solution, you will need to add 1 μL of DNA, 1 μL of Tol2 mRNA, and 6 μL of nano water (alternatively, you may wish to add 5 μL of nano water and 1 μL of a fluorescent lineage tracer, like 3% Texas Red, to better monitor which embryos received an injection). You will typically load injection needles with ~2–3 μL of injection solution.

For injecting DNA without the Tol2 transposase system:

- Prepare as above but do not add *tol2* transposase mRNA

For injecting lineage tracers:

- Dilute typically to 3% (w/v) with HPLC grade water. Aliquot a small sample into a 0.6 mL microcentrifuge tube as a working stock. Store at -20 °C in the lineage tracers box.

Pipette loading and calibration

1. Prepare your solution to be injected (above). If you will be injecting RNA, be sure to handle tubes, etc., with gloves. If you will be injecting lineage tracers such as fluorescein dextran, be sure to spin a small aliquot (1 min, max speed) to pellet any insoluble material that can clog the needle.
2. Inspect tip of a pulled capillary with filament (=injection needle) under the microscope to verify it is intact. Be careful in handling injection needles: if their tips enter your skin and break, they are small enough to travel through your blood stream and can cause embolism, etc.
3. Stick the needle vertically (tip down) onto a piece of modeling clay near the top edge of a 250 mL beaker. Using a pipette-loading tip, place ~2 μL of injection solution into the needle. You should load enough that the solution will ultimately fill the needle past the tapered “shoulder” portion, which helps to prevent medium in the dish being drawn into the needle by capillary action.
4. Wait 5–10 min until solution is drawn down to the tip by capillary action along the filament.
5. Turn on picospritzer. Set to 10–40 ms injection time, 10–20 psi.
6. Load needle onto needle holder, verifying that gasket is present and seal is effective. Take care not to break the needle in the holder. If a needle does break, make sure to remove all of the resulting glass fragments, which can otherwise clog future needles.
7. Under the microscope, *gently* scrape the needle tip on a metal object (e.g., razor blade, forceps). The opening should be too small to see under the microscope. Test if the tip has been broken by stepping on the picospritzer foot pedal and checking if fluid is expelled; if nothing comes out try again under mineral oil. If still nothing comes out, re-scrape the needle and verify that the needle has not been clogged with insoluble debris from your solution.
8. Calibrate the amount to be injected by inserting the needle tip into a pool of mineral oil on a depression slide. Adjust the injection time, psi, or both until a sphere of the desired diameter is produced. For Tol2 DNA/RNA injections you will typically want ~2 nL ≈ 156 μm diameter.
 - $\text{volume} = [4\pi (\text{diameter}/2)^3] / 3$
 - $\text{diameter} = 2 \sqrt[3]{(3 \times \text{volume}) / 4\pi}$
 - 1 nL = 10⁶ μm³
 - so, to inject 2 nL = 2 x 10⁶ μm³:
 $\text{diameter} = 2 \sqrt[3]{[3 \times (2 \times 10^6 \mu\text{m}^3) / 4\pi]} = 156 \mu\text{m}$
9. Place the loaded needle and needle holder out of the way.

sphere size—volume conversions	
diameter (μm)	volume (nL)
30	0.01
40	0.03
50	0.07
60	0.11
70	0.18
80	0.27
90	0.38
98	0.50
100	0.52
110	0.70
120	0.90
124	1.00
130	1.15
140	1.44
150	1.77
156	2.00
160	2.14
170	2.57
180	3.05
190	3.59
200	4.19

Embryo dechoriation, arraying, injection

1. In a 60 mm petri dish, collect newly fertilized embryos in 10% Hanks or system water. Pool clutches if several are available. The dish can be quite full.
 - For DNA constructs, RNA, or morpholinos, embryos should not yet have reached first cleavage.
 - For fluorescent lineage tracers, embryos should be as young as possible, but cells remain connected by cytoplasmic bridges through the ~16 cell stage allowing small molecules to become evenly distributed through this time.
2. Allow embryos to develop 10 min after they have been fertilized, and perform 3–5 below while you wait.
3. Load injection needle, if necessary, and calibrate the injections with mineral oil as above (I prefer to wait until I know I will have embryos).
4. Fill one 250 mL beaker with ~200 mL system water for each dish of eggs to be dechorionated. *Use glass beakers only as plastic will cause the embryos to stick.*
5. Dilute 0.5 mL of 20 mg/mL pronase into 10 mL of system water
6. Using a plastic pipette, remove and discard all solution from the egg dish and add 10 mL of diluted pronase. Swirl once or twice and incubate for 90 sec.
Note: this time and amount assumes you will be dechorionating several hundred embryos. If relatively small numbers of embryos will be dechorionated, working solutions of pronase should be scaled down accordingly to maintain a similar effective pronase concentration.
7. Immerse whole dish in the 250 mL beaker filled with system water and allow embryos to settle on the bottom.
8. Decant solution into a waste container, making sure that embryos do not touch the surface. Replace with system water by pouring the new solution *very gently* down the side of the beaker.
9. Allow embryos to sit 3 min.
10. Carefully decant the solution as above and replace once with new system water. Most chorions should fall off. Ideally the chorions will be lost easily so that embryos are not damaged by squeezing out of a partially digested chorion. If some chorions remain they will come off below.
11. Using a fire polished glass pipette, transfer the dechorionated embryos to an agar-lined 100 mm petri dish, the agar-lined injection dish, or a glass petri dish containing 10% Hanks or embryo medium.
Use only fire-polished glass pipettes. Plastic will stick to the embryos and glass edges that are not fire-polished can damage the embryos. Be gentle with pipetting: make sure embryos do not get compressed against each other at the pipette taper or upon exiting the pipette tip. Make sure the pipette opening is wide enough not to compress the chorions as you pull up the embryos. Use only glass or plastic dishes lined with agar, which is gentler on embryos than agarose.
12. If not already placed in one, transfer embryos to an agar-lined, bevelled injection dish so they are arrayed single file in the well of the dish. Set aside some embryos (e.g., ~30) as uninjected controls for fertilization success and overall viability.
13. Inject remaining embryos using the needle either hand-held or mounted on a micromanipulator (typically 2.0 nL solution per embryo). For RNA, DNA, or morpholino injections, it is important that the bolus of solution be injected into the cytoplasm of the first cell, not into the yolk. For low molecular weight fluorescent lineage tracers, solution can be injected into the yolk, as cytoplasmic streaming will allow it to diffuse to the cells of the embryo proper.
Make sure that solution is not flowing backwards into your needle when it is immersed! You can gauge this by examining the color of a lineage tracer if you have used one.

14. Once embryos have been injected, use the fire-polished pipette to move them to an agar-lined plastic 100 mL petri dish or glass unlined 100 mL petri dish containing 30 mL 10% Hanks or embryo medium and pen-strep (200 μ L pen-strep solution per 30 mL). Reload the injection dish with uninjected embryos and repeat.
15. Later in the day, sort embryos that are developing properly, placing them into new agar-lined dishes containing 30 mL 10% Hanks (or embryo medium) + 200 μ L pen-strep, with no more than 80 embryos/dish. Sort at the same time, or subsequently, for injection efficacy.
16. Sort again the following morning, placing surviving embryos into new agar-lined dishes containing 10% Hanks and pen-strep.
Embryos can be kept on un-lined plastic dishes beginning on day 2.

Cell transplantation

If many chimeras are to be made, it can be useful to disperse matched donor and host embryos at several different temperatures (e.g., embryo incubator, bench top) to change development rates and maximize the amount of time available for transplantation. You will need about one-third as many donor embryos as hosts (i.e., each donor can supply several hosts).

1. Under the microscope, inspect a pulled capillary without filament (= transplant needle; 1.0 mm outer diameter, 0.58 mm inner diameter; Harvard Instruments GC100-10, #30-0016) to make sure it has not been broken.
2. Mount the transplant needle on a needle holder designated for cell transplantation, which contains mineral oil and is connected to a mineral-oil filled, micrometer drive injection apparatus. Verify that the gasket is present and that a good seal has formed. Open the valve to the injection needle and add a small amount of pressure into the needle by turning clockwise the micrometer drive knob. Place the needle flat against the glass of the microscope stage and, using a razor blade, carefully break the needle so it is large enough for blastula stage cells to enter without being damaged. Ideally the needle should be free of jagged edges. Add pressure until mineral oil reaches the needle tip. If a needle breaks, make sure that no glass shards are left in the needle holder as these will clog future needles: if the needle breaks in the holder flush it the needle holder with mineral oil to ensure that any shards are washed out.
3. Take pre-made cell transplant dishes from the refrigerator so they warm to room temperature. Remove capillaries from the agar, creating slots that will hold the embryos.
4. To indicate the future positions of donor vs. host embryos, label the sides of cell transplant dishes with a Sharpie by placing a dot on the side closest to where the donor embryos will be loaded.
5. Flush the transplant dish with embryo medium to remove any stray pieces of agar.
6. Fill the dish with 10 mL embryo medium (recipe below). Add 150 μ L pen-strep.
7. When embryos have reached early-to-mid blastula stages, use a fire-polished pipette to load one row of donor embryos and three rows of host embryos per dish.
8. When embryos have reached mid-blastula stages (e.g., 3.3 hours post-fertilization) cells can be transplanted efficiently by drawing-up cells from one or more donors then gently placing them into hosts. Verify that cells are not being damaged during the transfer process. You should be able to see well-shaped cells in the needle and being expelled into the host; if you see cytoplasmic fluids or cellular fragments, the needle is too small and should be clipped (e.g., with forceps) further from the tip.
9. When transplants have been completed, allow the embryos to sit undisturbed for ~30 min, then gently transfer them to agar-lined 100 mm dishes containing 30 mL 10% embryo medium with 450 μ L pen-strep. Place no more than ~40–50 embryos per dish.

10. The following morning, sort for normally developing embryos, placing them into a fresh agar-lined dish containing 30 mL 10% Hanks with 250 μ L pen-strep. Place no more than ~40 embryos per dish. Embryos can be transferred to unlined dishes at 2 d, if desired.

Making agar lined plastic petri dishes

Note: We prefer to use reusable glass petri dishes for injected embryos. Make agar-lined dishes only if glass dishes are not available or for recovery of embryos after cell transplants. Melt a solution of 1% agar (*not* agarose) in 10% Hanks. Fill a 100 mm plastic petri dish about half way, then pour the agar into a new dish, leaving only a very thin (<0.5 mm) layer behind to cover the dish. Repeat until all the agar used. Do not pour thick dishes (as for bacteriological plates) as it wastes agar and reduces the volume of solution that can be added for holding the embryos. Wrap the dishes in saran wrap or their original sleeve for storage at 4 °C.

Making bevelled injection dishes

Into several sterile 100 mm bacteriological petri dishes, pour a thin layer of 1% agar (melted in 10% Hanks). Allow to set. Place a glass slide diagonally so that one side is resting on the edge of the dish and the other side is resting on the agar. Pour a second thin layer of agar. Allow to set. Remove slide. Wrap and store at 4 °C for use.

Making cell transplant dishes

Cut in quarters several capillaries used for transplantation (1.0 mm outer diameter, no filament). and place in a 60 mm dish for future use. Into several 60 mm sterile petri dishes, pour a thin layer of 1% agar (melted in embryo medium). Once set, place four pieces of capillary on the agar, each parallel to one another and separated by a few mm. Using a plastic pipette add a very small amount of agar to each dish so that each capillary is covered ~halfway. Allow to set, wrap, and store at 4 °C.

Hanks recipes: large volumes.

Hank's (full strength, 100%) stock solution^{1,2}

		g per 10 L solution ³
sodium chloride (NaCl)	0.137 M	80.0
potassium chloride (KCl)	5.4 mM	4.0
sodium phosphate, dibasic (Na ₂ HPO ₄)	0.25 mM	0.358
potassium Phosphate, monobasic (KH ₂ PO ₄)	0.44 mM	0.6
calcium chloride (CaCl ₂)	1.3 mM	1.44
magnesium sulfate (MgSO ₄ •7H ₂ O)	1.0 mM	2.46

¹ working strength is 10%

² all salts are anhydrous except as indicated

³ pH to 7.5 with NaOH or HCl

10% Hank's, for routine embryo rearing

- combine 9 parts RO water to 1 part Hanks full strength stock solution
- mix well

Hanks and embryo medium: small volumes

Hanks stock solutions* (for small amounts of working strength solution or Embryo Medium)

Stock #1	Stock #2	Stock #4	Stock #5	Stock #6
<ul style="list-style-type: none"> • 8.0 g NaCl • 0.4 g KCl • in 100 ml H₂O 	<ul style="list-style-type: none"> • 0.358 g Na₂HPO₄ • 0.60 g KH₂PO₄ in 100 ml nano H₂O 	<ul style="list-style-type: none"> • 0.72 g CaCl₂ in 50 ml nano H₂O 	<ul style="list-style-type: none"> • 1.23 g MgSO₄ • 7H₂O in 50 ml nano H₂O 	<ul style="list-style-type: none"> • 0.35 g NaHCO₃ 10.0 ml dd H₂O

* there is no stock #3; all salts are anhydrous except as indicated

Hank's Premix	Hank's (full strength)	Embryo medium for injections, cell transplantation, etc.
Combine the following in order: 10.0 ml Solution #1 1.0 ml Solution #2 1.0 ml Solution #4	9.9 ml Hank's Premix 0.1 ml Stock #6	1.0 ml Hank's Stock #1 0.1 ml Hank's Stock #2 1.0 ml Hank's Stock #4 95.9 ml dd H ₂ O 1.0 ml Hank's Stock #5 1.0 ml fresh Hank's Stock #6 Use about 10 drops 1 M NaOH to pH 7.2