Chick Neural Tube Electroporation

(Protocol by Ben Allen)

Updated 110409

This protocol is compiled from previous protocols, notes, and discussions with Toyoaki Tenzen and Yu Wang.

I. Needle preparation

1. Generate needles for DNA injection using a Sutter Instrument Co. model P-87 Flaming/Brown micropipette puller as follows:
   Program conditions: Heat = 400, Velocity = 120, Pull = 140, Time = 145
   N.B. These conditions may need to be adjusted to achieve optimal needles.

Needle puller is located in Zebrafish Facility (Vivarium, Room D621).

2. Use FHC borosilicate capillary tube w/ omega dot fiber (1mm O.D. x 0.5mm I.D.; cat. #3030-1).

3. Store needles in 150mm dish, using strips of modeling clay as a needle holder.

II. DNA preparation

1. Perform Qiagen Plasmid Midi Prep of appropriate pCIG constructs (see Qiagen protocol for detailed instructions).
   (N.B. DNA should be freshly prepared before electroporations- use of DNA prepared more than 2 weeks before electroporation will result in lower GFP expression).

2. Ethanol precipitate DNA as follows:
   50µg DNA in 50µl TE Buffer, pH8.0
   5µl 3M Sodium Acetate, pH5.2 (1/10 volume)
   125µl 100% EtOH (2.5 volumes)
   Incubate for 1hr to overnight at -80°C.
   (N.B. DNA should visibly precipitate immediately after mixing).
   Centrifuge at 13,000 x g for 10min at 4°C to pellet DNA.
   Aspirate 100% EtOH.
   Wash DNA with 200µl 70% EtOH.
   Centrifuge at 13,000 x g for 10 min at RT.
   Aspirate 70% EtOH.
   Air dry pellet for 5-10min at RT.
   Resuspend pellet in 50ul 1X sterile PBS (w/Ca++ and Mg++; pH7.4) to a concentration of 1µg/µl.
   Add 5µl of 1% Fast Green Solution.

3. Spin DNA/Fast Green mix at 13,000rpm for 1min at RT to pellet any precipitate.

4. Add ~5ul of DNA/Fast Green mix to a needle using Eppendorf microloader pipette tips (cat. #930001007). Load at least three needles for each DNA construct (Number of needles needed will vary depending on the number of eggs to electroporate).

5. Store needles vertically on the electroporator to allow DNA to load into the tips of the needles (again using modeling clay as a needle holder).

III. Egg preparation

1. Order eggs from Michigan State University as follows:
   Egg Orders:
   Angelo Napolitano (517-355-0360; napolit3@msu.edu; $7.50/12 eggs)
   Egg Delivery:
   Sebastian @ Metro Delivery (734-973-0973; $36/delivery)
   N.B. Try to coordinate deliveries with Fumi Ebisu in Kate Barald's lab.

Day 1:

1. Following egg delivery, unpack and store at RT until ready to begin incubation. **N.B. Find a location with constant, cooler temperature (i.e away from sunlight) for ideal storage conditions.**

2. Turn on 37°C incubator (GQF Model #1550 Hatcher), add sterile water to moisture pan (or use Automatic water system) to humidify the incubator.

3. Arrange eggs horizontally, and number on side with permanent marker.

4. Incubate eggs at 37°C for approximately 42 hrs. (e.g. eggs incubated starting at 3pm on Tuesday will be ready for electroporation around 9am on Thursday). Ideal stage for electroporation is HH stage 11.

Day 3:

1. Using a sterile 18g needle attached to a 10ml syringe, insert the needle at a sharp downward angle through shell (carefully) at blunt end of egg. **N.B. After lying horizontally, the embryo will be positioned at the top of the egg.**

2. Withdraw 5-10ml of albumin (egg white) from egg, being careful not to withdraw any of the yolk. **N.B. This will reduce the volume of fluid in the egg, preventing loss of the embryo during the windowing process.**

3. Using packing tape, cover the hole generated by the needle, as well as the entire top of the embryo (this will prevent cracking of the shell during subsequent windowing of the embryo).

4. Window embryos by removing an approximate 1in. diameter circle of shell from the top of the egg using scissors. **N.B. Only window 5 embryos at any one time to prevent drying out of embryos during electroporation.**

5. Dilute 1% Neutral Red Solution ~ 1:10 in sterile PBS w/Ca^{++} and Mg^{++} (PBS-CM).

6. Add several drops on top of embryo to counterstain. Embryos will become significantly more visible within 1-2 minutes.

IV. DNA Injection and Electroporation

1. Set up electroporation apparatus as follows:
   Position electrodes 5mm apart.
   Attach needle to stainless steel needle holder, which is connected to a PLI-90 injector supplied with compressed Nitrogen gas for injections.
   Break needle tip under the microscope to generate a hole of appropriate size for injections. **N.B. If the hole is too small, not enough DNA will load, if the hole is too big, all the DNA will be expelled from the needle.**
2. Add several drops of 1X PBS-CM to the embryos to prevent drying out.
3. Position embryo horizontally, with head to the right side of the field of view.

4. Insert needle into lumen of neural tube, being careful not to puncture too deeply into the embryo.
5. Inject DNA into lumen of neural tube using PLI-90 injector, until green dye is visible throughout the neural tube. **N.B. Nitrogen gas pressure should be ~10psi at tank regulator, and 4psi at injector regulator. Use 2 second injection pulses during loading of DNA.**
6. Remove needle and position electrodes on either side of the embryo, just caudal to the developing heart.
7. Overlay electrodes with 1X PBS-CM to prevent drying out.
8. Electroporate embryo using BTX ECM 830 electroporator with the following settings:
   Voltage: 20V (24V actual), Pulse length: 50msec, Pulse interval: 950msec, Pulse number: 5
9. Remove electrodes, overlay embryos with a few additional drops of 1X PBS-CM, cover embryos with tape, and place in GQF 1550 incubator.
10. Incubate embryos for approximately 48hrs in incubator; remove at HH stage 21-22.
11. Discard dead embryos (live embryos have well developed vasculature and clearly visible beating hearts); dissect live embryos and process for immunofluorescence analysis.

12. Embryo collection and processing

1. Live embryos can be screened for GFP/RFP expression in ovo, or can be dissected out, for subsequent fluorescent screening, using a Nikon SMZ1500 stereomicroscope.
2. To dissect out embryos, remove tape and cut vertical slits in egg shell.
3. Crack egg over a 100mm dish, such that the embryo remains on top of the yolk.
4. Use scissors to cut away tissue surrounding embryo.
5. Transfer embryo to a fresh dish containing 1X PBS-CM using a transfer pipet.
6. Dissect away extraembryonic tissue.
7. Fix embryos for 1hr in 4% PFA on ice.
8. Wash embryos 3 x 5min with 1X PBS-CM.
9. Incubate embryos on rocker at 4°C overnight in 1X PBS-CM + 30% sucrose
10. Embed embryos in OCT, and freeze in dry ice/EtOH bath.
11. Store embryos at -80°C until ready to section.
12. Cut 12µm sections on a cryostat and perform immunofluorescent analysis (see mouse neural tube IF protocol for details).
13. Egg waste should be frozen overnight at -20°C prior to disposal in carcass cooler in Animal Facility (Vivarium, Room D050).

1. Solutions, materials and other notes

1% Fast Green Solution
100mg Fast Green FCF (Sigma cat. #F7252)
10ml 1X PBS (w/Ca++ and Mg++; pH 7.4)
Dissolve Fast Green in PBS.
Filter through 0.2µm syringe filter into 15ml conical tube.
Store at RT.

1% Neutral Red Solution
500mg Neutral Red (Sigma cat. #N6634)
50ml 1X PBS (w/Ca++ and Mg++; pH7.4)
Dissolve Neutral Red in PBS.
Filter through 0.2µm syringe filter in 50ml conical tube.
Store at RT.