Chapter 8

The Use of Chick Embryos to Study Wnt Activity Gradients

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Abstract

The chick spinal cord provides a valuable model for assessing Wnt signaling activity. Loss or gain of function constructs that are transfected by electroporation can be directed to a single side of the spinal cord, thus leaving the contralateral side as an internal control. Here, we describe a method for measuring Wnt signaling via the use of BAT-Gal, a β-catenin dependent Wnt reporter.

Key words WNT1, WNT3A, β-catenin, BAT-Gal, Electroporation, Gradient, Spinal cord, Chick, In ovo

1 Introduction

Wnts are secreted morphogens that act in a concentration-dependent manner [1, 2]. Disruption of Wnt gradients is implicated in developmental defects and in cancer [1, 2]. Thus, understanding the formation of vertebrate Wnt gradients is of great interest. We use the developing chick spinal cord as a biologically relevant three-dimensional tissue for the study of vertebrate Wnt gradient formation. Proper development of the spinal cord requires a dorsal to ventral gradient of WNT1 and WNT3A for outgrowth and patterning [3–5]. As it has not yet been possible to directly detect any Wnt protein gradient in vertebrates, researchers currently rely on visualization of the Wnt activity gradient [6]. Our lab has developed a number of protocols to assay for WNT1/3A activity and gradient formation [6–9]. These include measuring (1) the relative percent of Islet-1 positive DI3 interneurons and motor neurons [8, 10], (2) the relative percentage of cells in late G2/M phase as indicated by immunostaining with anti-phosphohistone H3 [6–9], (3) the expression domain of FZD10 [7, 11], and (4) activation of the β-catenin dependent BAT-Gal reporter [6, 12]. All of these assays take advantage of our ability to electroporate gain and loss of function constructs into the spinal

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cord after neurulation. However, the BAT-Gal reporter represents the most specific readout of β-catenin dependent Wnt signaling. A detailed protocol for electroporating DNA constructs, such as a WNT1 expression construct and the BAT-Gal reporter, into the spinal cord is presented below.

2 Materials

Prepare all solutions with ultrapure water. In our lab, we use distilled deionized water. These experiments require fertile eggs, purified DNA, a pneumatic injector, a micromanipulator, platinum electrodes, and a square pulse electroporator. Follow all waste disposal regulations when disposing of waste materials. Note that formaldehyde (the monomeric version of paraformaldehyde) and sodium azide are hazardous. Please check the Safety Data Sheet and handle with care. Disposal should be carried out according to institutional protocols.

2.1 Electroporation

1. Fertile chicken eggs are purchased from a local hatchery (Petaluma Farms) (*see* **Note 1**). If you are in a real pinch, it is possible to use fertile eggs from the grocery story so long as you buy them on the day they are delivered to the store. We find that storing eggs at room temperature is fine in the cool environment of San Francisco. In regions with warmer temperatures, it may be necessary to store them at 4 °C. It is best to use eggs within 1 week. It is not necessary to use pathogen-free eggs.
2. Tyrode's solution can be commercially purchased or prepared in the lab. To prepare Tyrode's, weigh 265 mg $CaCl_2{\cdot}2H_2O$, 214 mg $MgCl_2{\cdot}6H_2O$, 200 mg KCl, 1 g $NaHCO_3$, 8 g NaCl, 50 mg NaH_2PO_4 (anhydrous), 1 g d-glucose (dextrose). Add water to 800 ml and then adjust pH to 7.4 using 6 M HCl. Increase volume to 1 L, recheck pH, and adjust if necessary. Sterilize by filtration through a 0.2 μm filter; do not autoclave.
3. 5× Phosphate buffered saline, pH 7.4 (PBS): Weigh 40 g NaCl, 1 g KCl, 13.6 g $Na_2HPO_4{\cdot}7H_2O$, and 1.2 g KH_2PO_4. Add water up to a volume of 800 ml. Adjust pH with 7.4 using 5 M NaOH or 6 M HCl. Increase volume to 1 L, recheck pH and adjust if necessary. Sterilize by autoclaving. 5× PBS can be diluted to 1× PBS with water.
4. Pelikan India Ink (MisterArt): Dilute 1 ml of India ink to 50 ml using Tyrode's solution.
5. 1× Penicillin/Streptomycin (P/S): Dilute 0.5 ml of 100× P/S to a final volume of 50 ml using Tyrode's solution.
6. 6× Fast Green FCF (Fisher) is prepared at a concentration of 2 mg/ml in 1× PBS. Weigh 2 mg of Fast Green and dissolve in 1 ml 1× PBS.

7. 2% Carboxymethylcellulose sodium salt: Weigh 200 mg carboxymethylcellulose. Add water to a volume of 10 ml. Mix and store at room temperature.
8. 2:1 solution of Fast Green Dye and carboxymethylcellulose. Add 666 µl of 6× Fast Green Dye to 333 µl of 2% carboxymethylcellulose. Store at room temperature.
9. Purified DNA: The BAT-Gal reporter is used in combination with gain or loss of function constructs to specifically measure β-catenin dependent Wnt signaling (Fig. 1a, [12]). pCIG is used the base vector for most of our gain of function studies. In this vector, a β-actin promoter with a CMV enhancer drives the expression of a bicistronic transcript encoding a protein of choice and a nuclear variant of eGFP (Fig. 1b, [4]). A variant of psiSTRIKE in which hmgGFP has been replaced with a nuclear variant of eGFP is used for our loss-of-function studies (*see* **Note 2**).
10. DNA constructs can be purified using any of a variety of kits. We use a midi prep kit to purify DNA from 50 ml cultures using the instructions provided with the kit. Briefly, DNA pellets are resuspended in 30 µl of H_2O and incubated overnight at RT. The following day, the DNA solution is centrifuged at 15,000 × *g* before transferring supernatant to a new tube. We find that this extra step helps sediment any particulate matter and thus, prevents needles from clogging. The concentration is determined by spectrophotometry at 260 and 280 nm. Ideally, DNA should be between 4 and 9 mg/ml. DNA is stored in

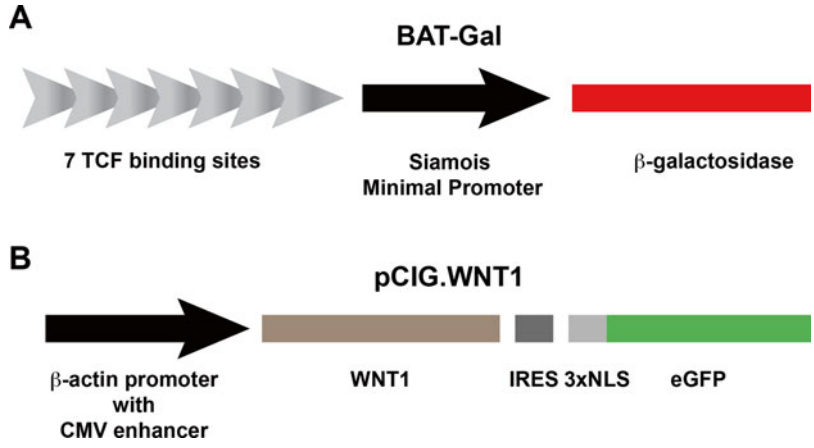

Fig. 1 Constructs used to detect Wnt signaling in the chick spinal cord. (**a**) 7 TCF sites positioned upstream of a minimal Siamois promoter are used to drive the expression of β-galactosidase [12]. (**b**) BAT-Gal can be co-electroporated in combination with any of a variety of loss or gain of function expression constructs. Here we depict the salient features of pCIG.WNT1 [4, 6]. Briefly, a β-actin promoter (with a CMV enhancer) is used to drive the expression of a bicistronic transcript encoding chick WNT1 and a nuclear variant of GFP (NLS = nuclear localization sequence). An internal ribosome entry site (IRES) allows the two proteins to be translated separately

2.5 μl aliquots at −80 °C. Immediately prior to electroporations, mix 1 μl of the 2:1 solution of 2% methylcellulose and 6× Fast Green (2 mg/ml in PBS) with 2.5 μl purified DNA.

11. Dissection tools—small dissection scissors and Student Dumont #5 forceps.
12. Syringes and needles—one 10 ml syringe with a 25 gauge needle; one 10 ml syringe with an 18 gauge needle.
13. Tungsten dissecting needle—We sharpen our dissecting needles using a modification of a technique first reported in 1965 [13]. A short piece of 0.13 mm diameter tungsten wire is connected to a toothpick and then electrolytically sharpened. To sharpen this blade, first connect the positive lead to the tungsten wire and then immerse it in 0.5 M KOH. The negative lead should be connected to a short copper wire, which is also submerged in the KOH solution. We gradually turn up the voltage to 5–10 V to sharpen the tungsten wire. Mild bubbling is to be expected, but really vigorous bubbling should be avoided.
14. Injection needles—We use a Sutter P-97 Micropipette needle puller to make needles for the injection of DNA into the spinal cord. We use thin wall borosilicate tubing with a filament and a 1.0 mm outside diameter (Sutter). The settings are variable depending on the filament in use. However, our typical settings are 75 Pull, 200 Velocity, and 80 Time. The "Heat" setting is determined by first running a ramp test. This program will generate a needle with a closed tip. The tip can be broken with forceps after loading with DNA. Needles can be pulled well in advance of an experiment and stored in a 150 mm petri dish with two strips of clay in the bottom. We use microloader tips to backfill needles with DNA solution.
15. Electrodes—While it is possible to purchase electrodes from commercial vendors, such as the Protech platinum z-shaped blunt needle electrode (CUY611 or 613) or BTX Genepaddles, it is much more economical to make your own. We construct our electrodes using a slight modification of the protocol outlined by Krull [14]. After pre-warming the soldering iron, we use lead-free solder to connect a 4–5 cm piece of platinum wire (Fig. 2c, 0.01 in. diameter platinum rod; A-M Systems, cat #711000) to the pin stamped brass (Fig. 2b, Digikey). It is important to use the minimal amount of solder. This process should be repeated to make a second electrode (Fig. 2d). Next, we solder red stranded 22 gauge wire (Newark Electronics) onto one gold-plated jack/socket (Newark Electronics) and black stranded 22 gauge wire (Fig. 2a; Newark Electronics) onto a second gold-plated jack/socket. The length of the red and black wire should be sufficient to comfortably span the distance from the electroporator to the egg. We then clip off

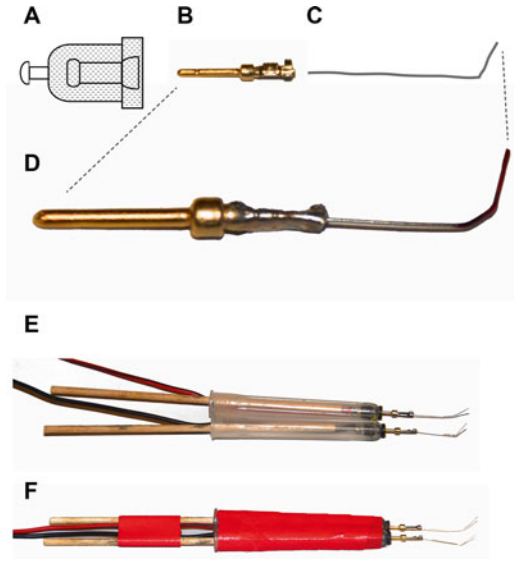

Fig. 2 Construction of electrodes. (**a**) Gold plated jack/socket. (**b**) Brass stamped pin. (**c**) Platinum wire (it can be bent before or after soldering). (**d**) A magnified image of the platinum wire that has been soldered to the brass stamped pin. The gold-plated jack/sockets are then soldered to the *black* and *red wires* (not shown). (**e**) This image shows how the gold plated jack/socket (*black*) is inserted into the cut end of a needle cover. A wooden handle is made by inserting the wood from a long cotton swab (with the cotton removed) into the opposite end of the needle cover. (**f**) The entire assembly is taped together with lab tape (*red*) to stabilize the configuration

the closed end of two needle covers (from 25 gauge needles). Take the free end of the red wire and pass it through the newly clipped end of the needle cover (from right to left in Fig. 2e) to secure the gold plated/socket in the end of the needle cover (only the lip of the gold plated/socket should remain outside the needle cover). This step is repeated for the black wire. We then make a handle by inserting two long sticks (from long cotton swabs) into the back end of the needle cover (Fig. 2e). We then use tape to seal the whole assembly (Fig. 2f). The red and black wires are then connected to a banana connectors with male ends (available at any electronics store). The soldered pin stamped brass/electrode assemblies should then be inserted into the gold plated jack/sockets. Use forceps to bend the ends of the electrodes (approximately 0.5 cm from the end); the electrodes are now ready for use.

16. Micromanipulator: We use a MM33 right micromanipulator (Sutter).
17. Injector: PV820 Pneumatic PicoPump with foot pedal World Precision Instruments hooked to a gas N_2 tank and needle holder is fitted with the green gasket (holds 1.0 mm needles) (*see* **Note 3**).

18. Electroporation system: There are many available square wave electroporation systems on the market. We use the ECM 830 Square Wave Electroporation system made by BTX (*see* **Notes 3** and **4**).

2.2 Embedding and Sectioning

1. 4% paraformaldehyde: 30 ml 1× PBS is added to 10 ml 16% paraformaldehyde and cooled on ice.
2. Embedding solutions: 15% sucrose in 1× PBS containing 0.01% NaN_3, 30% sucrose in 1× PBS containing 0.01% NaN_3, 60% sucrose in PBS:OCT (1:1) containing 0.01% NaN_3 and Tissue Tek OCT (VWR).
3. Histology molds: Peel-A-Way Disposable Embedding Molds Truncated 22 × 22 mm (VWR).
4. Glass slides and coverslips: VWR Superfrost Plus Microslide (VWR), Fisherfinest Premium Cover Glasses No. 1 (Fisher).
5. Mounting Solution: SlowFade Gold Antifade (Molecular Probes).
6. Cryostat: We use a Leica CM 1950 cryostat.

2.3 Immunostaining

1. Blocking buffer: 3% lamb serum in 1× PBS containing 0.1% Tween.
2. Antibodies—anti-β-galactosidase J1E7 (Developmental Studies Hybridoma Bank, supernatant diluted 1/100 in blocking buffer) and goat anti-mouse IgG(H + L)-Cy3 (Jackson ImmunoResearch, diluted 1/200 in blocking buffer).

3 Methods

All procedures should be carried out at room temperature unless otherwise specified.

3.1 Incubating Eggs

Eggs should be incubated on their side in a humidified egg chamber 39 °C. If available, it is preferable to use an incubator with egg turning capability as this will increase the viability of the embryos.

The Hamburger and Hamilton staging guide provides an estimate of the time needed for embryos to develop to certain stages [15]. Note that some seasonal variability is not unusual. Depending on the experiment, we electroporate embryos ranging from HH stage 12 to 17.

3.2 Loading Needles

1. Long microloader tips are used to backload needles with 3.5 µl of the DNA solution containing fast green dye and CM-cellulose.
2. After loading, the tip of the needle is clipped off using forceps.

3.3 Windowing Eggs

1. After the appropriate incubation time, eggs are removed from the incubator.
2. Small petri dishes can be modified with colored lab tape to make a suitable egg holder for injections.
3. Before windowing eggs, a small piece of packing tape should be adhered to the side of the egg that is facing up (Fig. 3a) (*see* **Note 5**). We use an 18 gauge needle to remove 5–7 ml of egg white from the large end of the egg. After gently penetrating the eggshell with the scissors, the needle should be angled down, bevel side down, to avoid puncturing the yolk.
4. Small dissection scissors are then used to cut a small circle in the eggshell on the taped side of the egg (Fig. 3b). The cut-out piece of shell can be discarded.

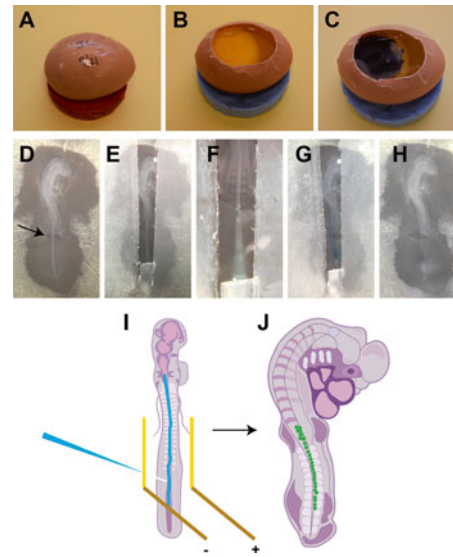

Fig. 3 Electroporation procedure: Here, we outline the basic steps of the electroporation. (**a**) The top of the egg is covered with packing tape to prevent the shell from cracking while cutting the window. A windowed egg is shown before (**b**) and after (**c**) the injection of India ink underneath the embryo. (**d**) An *arrow* demarcates the position of a small hole torn in the vitelline membrane. (**e**) The embryo is overlaid with a piece of Parafilm such that the spinal cord remains exposed. (**f**) The needle is oriented parallel to the spinal cord of the embryo and inserted into the spinal cord at the level of the segmented plate. (**g**) After injection of the DNA, the fast green dye is visible in the lumen of the spinal cord and extends up to at least the level of the heart. (**h**) After electroporating the embryo, the Parafilm is removed and the embryo is allowed to recover. In (**i**) and (**j**), we show a graphic of the overall electroporation procedure. (**i**) DNA (*blue*) is injected prior to the electroporation. (**j**) After 16–48 h of incubation, GFP is expressed on the right side of the spinal cord

3.4 Injecting and Electroporating

1. India ink diluted in 1× Tyrode's is then injected under the embryo using a 25 gauge needle connected to a 10 ml syringe. The needle should be inserted just outside the blood islands at a nearly horizontal angle, bevel side up. Ink should be injected until the embryo is clearly visible (Fig. 3c).
2. Stage embryos according to Hamburger and Hamilton [15].
3. We use an electrolytically sharpened tungsten needle to make a small tear in the vitelline membrane at the level of the segmental plate or most recently formed somites (Fig. 3d).
4. Parafilm cut in the shape of a "U" is first dipped in 1× P/S in Tyrode's solution and then placed over the embryo, leaving the spinal cord exposed. This protects the embryo from being scorched during the electroporation (Fig. 3d).
5. The embryo should be aligned such that the needle is oriented parallel with the spinal cord of the embryo (Fig. 3f).
6. The needle should be inserted into the posterior spinal cord where the vitelline membrane was torn (Fig. 3f). DNA should be injected until the entire lumen is full of DNA. The fast green dye in the DNA solution ensures that it is easily visible. The spinal cord should be filled from the posterior most end up to at least the level of the heart (*see* Fig. 3g). Once sufficiently full, retract the needle completely and rotate the egg 90° so that the tail of the embryo is orientated to the bottom of the stage.
7. Two to four drops of 1× P/S in Tryode's solution is added on top of the embryo and Parafilm.
8. Electrodes are then quickly placed on either side of the embryo on top of the Parafilm (Fig. 3i, *see* **Notes 6** and **7**). We use four pulses at voltages between 48 and 55 with a 50 ms pulse length (*see* **Note 8**). The voltage used depends on the stage of embryos and the electrodes used. Smaller embryos (HH stages 12–14) require lower voltage while larger embryos (HH stages 15–17) require higher voltage. We aim to use a voltage at which the majority of the embryos are successfully transfected and up to 25 % are dead (*see* **Note 9**).
9. After electroporation, the embryos are overlaid with an additional 1 ml of 1× P/S before removing the Parafilm (Fig. 3h) and resealing the eggs with packing tape. Eggs should be returned to the incubator with no turning (*see* **Note 10**).

3.5 Harvesting and Embedding Embryos

1. Between 16 and 48 h later, embryos are screened for GFP fluorescence using an Olympus SZX12 dissecting scope outfitted with a UV light source (in ovo). Successfully transfected embryos are then harvested for further analysis (Fig. 3j) (*see* **Note 10**). Note that embryos must be allowed to develop to at least HH stage 21 to effectively visualize the Wnt gradient

utilizing the Islet-1 or the phosphohistone H3 method for measurement. For the activation of the BAT-gal reporter HH stage 18 is sufficient.

2. When harvesting embryos, the extraembryonic tissue should be trimmed from embryo. However, it is not necessary to remove all of the tissue as it will not interfere with sectioning. The forebrain and hindbrain should be punctured with forceps to allow efficient infiltration of OCT. Alternatively, the entire head can be cut off.
3. Embryos are fixed in 4% paraformaldehyde prepared in 1× PBS for 1 h at 4 °C on a nutator.
4. Embryos are moved sequentially from 15% sucrose in PBS containing 0.01% NaN_3 to 60% sucrose in PBS:OCT (1:1) containing 0.01% NaN_3 at 4 °C on a nutator. Embryos are then equilibrated in OCT for 30 min at room temperature on a rotator prior to embedding.
5. Embryos are embedded in disposable histology molds. For transverse sections, we orient the embryo with its tail at the bottom and the head at the top. Once embryos are oriented, the block is frozen in a bath of dry ice and EtOH. Blocks can be stored at −80 °C.
6. We cut 10–20 μm sections using a Leica CM1950 Cryostat. We usually collect sections on 5–10 slides such that each slide has adjacent serial sections. For instance, section 1 goes on slide 1 of 10 while section 2 is placed on slide 2 of 10. We return to slide 1 of 10 for section 11. We usually make three rows of sections with 5–10 sections per row.

3.6 Immunostaining Embryos

1. Remove OCT from the slides by soaking in 1× PBS at room temperature for 10 min and then subsequently washed two additional times in 1× PBS (can be done a slide mailer).
2. Block slides (in blocking buffer) for 1 h in a humidified chamber (a plastic box with wet paper towels; the slides rest on two trimmed serological pipettes held in place by clay or tape).
3. Incubate slides in primary antibody diluted in blocking buffer overnight at 4 °C in a humidified chamber. The following day, slides are wash four times in 1× PBS containing 0.1% Tween in a quadriPERM dish (Four well plate, Greiner Bio-One).
4. Block slides (in blocking buffer) for 30 min at room temperature.
5. Incubate with secondary antibody for 2 h at room temperature. Protect from light from this point forward.
6. Wash four times with 1× PBS containing 0.1% Tween in a quadriPERM dish.

7. Post-fix slides in 4% paraformaldehyde diluted in 1× PBS in a slide mailer.
8. Wash in two times in 1× PBS containing 0.1% Tween in a quadriPERM dish.
9. Add 75–125 µl of SlowFade to the slide and carefully place coverslip on top.
10. Using a vacuum attached to a pipet tip to gently remove any excess SlowFade and then seal the edges of the coverslips with fingernail polish. Allow to dry before imaging.

3.7 Imaging Embryos

1. Confocal Microscopy: For confocal microscopy, specimens are excited using the 488-nm line of an argon ion laser for visualization of GFP and the 543-nm line of a green helium–neon laser for visualization of Cy3. We capture fluorescence emission on a Zeiss LSM710 confocal microscope with a 40× oil objective at 0.6× digital zoom (Fig. 4).
2. Image Processing: We use Adobe Photoshop for final image data processing.

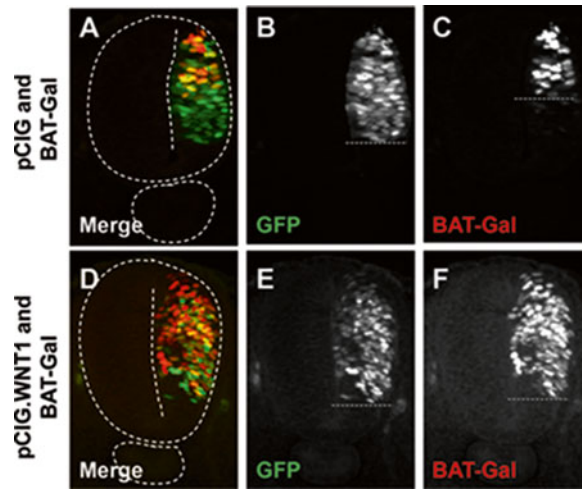

Fig. 4 Visualization of Wnt signaling in the developing chick spinal cord: HH stage 12–14 chick embryos were co-electroporated with pCIG (GFP alone) and BAT-Gal (**a**–**c**) or pCIG.WNT1 (GFP and WNT1) and BAT-Gal (**d**–**f**) and harvested at HH stage 18. The GFP expression (*green*) marks successfully electroporated cells. The activation of the BAT-Gal reporter is detected by immunostaining with anti-β-galactosidase antibody followed by anti-mouse secondary coupled with Cy3 (*red*). The spinal cord and notochord are outlined in (**a**) and (**d**). The *dashed lines* in (**b**–**c**, **e**–**f**) provide a comparison of the ventral most boundary of GFP and BAT-Gal. In (**a**–**c**), endogenous Wnt signaling is restricted to the dorsal half of the spinal cord while in (**d**–**f**), the overexpression of WNT1 causes the domain of Wnt signaling to fill the entire transfected region (as detected by GFP)

4 Notes

1. We use Rhode Island Red eggs for our electroporations (based on availability in our area); however, most chicken sequence information is derived from White Leghorns. While we would predict that small differences in sequence would have no significant effect on gain of function studies, it is possible that there could be ramifications for knockdown studies. Thus, if you choose to use a strain other than White Leghorn for knockdown studies, we recommend confirming the sequence of the gene you wish to target.
2. A variety of electroporation constructs are available that express a variety of fluorescent proteins. We find that the use of a nuclear localized variant of GFP (or whatever fluorescent protein you choose) is particularly useful as it allows us to use the NIH Image J Image Based Tool for Counting Nuclei to quantitate the total number of cells successfully transfected.
3. Foot pedals are highly useful for controlling the pneumatic injector and the electroporator. Using them frees up one hand for the micromanipulator and the other to hold the electrodes.
4. Though electroporations require some specialized equipment, the technique is accessible to all kinds of researchers, including undergraduate students. It typically requires 2–3 weeks for undergraduates in the lab to become proficient at electroporations.
5. The adhesive in packing tape can be toxic to the embryos. Therefore test the packing tape first on windowed eggs or use 3 M—Scotch® 3750 Packaging Tape—3″.
6. When electroporating embryos, depress the electrodes lightly to submerge the electrodes. By depressing electrodes equally, the DNA will be distributed along the entire dorsal/ventral extent of the spinal cord. By depressing one electrode more than the other, one can direct DNA to the dorsal or ventral spinal cord.
7. Electroporation should be carried out as quickly as possible after injecting DNA into the spinal cord.
8. Bubbles should be visible on the negative electrode after a successful electroporation. If no bubbles are observed, it is possible that no voltage was applied to the embryo. Ensure that electrodes are properly connected to power supply.
9. If electroporations are still not successful, we recommend starting with freshly prepared DNA and increasing the voltages used for the electroporation.
10. We find that although autoclaving egg waste is not required, it reduces unpleasant odors.

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