

# Spatially and temporally controlled electroporation of early chick embryos

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**The introduction of *in ovo* electroporation a decade ago has helped the chick embryo to become a powerful system to study gene regulation and function during development. Although this is a simple procedure for embryos of 2-d incubation, earlier stages (from laying to early neurulation, 0–1 d) present special challenges. Here we describe a robust and reproducible protocol for electroporation of expression vectors and morpholino oligonucleotides into the epiblast of embryos from soon after laying (stage XI) to stages 6–7 (early neurulation), with precise spatial and temporal control. Within 3 h, about 12 embryos can be electroporated and set up for culture by the New technique; the effects of morpholinos can be assessed immediately after electroporation, and robust overexpression from plasmid DNA is seen 2–3 h after electroporation. These techniques can be used for time-lapse imaging, gain- and loss-of-function experiments and studying gene regulatory elements in living embryos.**

## INTRODUCTION

The chick embryo has long been the vertebrate system of choice for experimental embryology, particularly for grafting cells and tissues and for lineage mapping<sup>1</sup>. In the last decade, it has been made more powerful by the discovery that *in vivo* electroporation can be used very efficiently to introduce expression vectors<sup>2,3</sup> and small interfering RNA constructs<sup>4–6</sup> particularly into the neural tube at around day 2 of development, for gain- and loss-of-function approaches. At this stage, the neural tube is a tight, high-resistance columnar epithelium with a closed lumen. DNA can be injected into the lumen and electroporation carried out: electrodes are placed on each side of the neural tube and pulses of voltage applied across them. This has allowed specific locations in the embryo to be targeted at precise times during development. Using these techniques, it has also been possible to electroporate embryos from stage 4 (ref. 7) onward quite effectively<sup>8–13</sup>. However, embryos during the first day of development are much more difficult to electroporate because they are more delicate and because the magnitude of the current required to introduce constructs into the epiblast (a much less tight epithelium than the neural tube<sup>14</sup>) seriously damages the cells; this problem becomes increasingly acute the earlier the stage of the embryo. Until recently only two studies<sup>15,16</sup> had reported successful electroporation of plasmid DNA at early and pre-gastrulation stages for embryos in EC culture<sup>17</sup>. However, the success of electroporation and embryo survival is still low<sup>15,16</sup> and the method does not allow spatially targeted delivery of the plasmid. To overcome these problems, as well as to offer a method that allows targeted delivery of morpholino oligonucleotides (MOs) in addition to DNA plasmids, we have developed a method based on New culture<sup>18</sup> which allows precise targeting and virtually 100% survival of embryos for at least 24 h. A full demonstration of this method was published recently<sup>19</sup>.

Here, we provide a general, robust and reproducible protocol for electroporation of young embryos, which allows targeting of plasmid DNA or MO—either to a very precisely selected small region or to a very large area of the embryonic epiblast—at any stage between pre-streak (stages XI–XII, ref. 20) and early neurula (stage 7, ref. 7). In addition to gain- and loss-of-function

approaches, these techniques can be used for time-lapse imaging (e.g., by introducing DNA encoding fluorescent proteins, which can be targeted to the nucleus or to the membrane<sup>19</sup>) as well as for studying the activity of gene regulatory elements<sup>9,11,21</sup> in living embryos.

## Experimental design

**Choice of system: *in ovo*, New culture or EC culture?** Although at neural tube stages, electroporation is most easily achieved with the embryo *in ovo*, this is virtually impossible before stages 7–8 (ref. 7). The main reason for this is that, before these stages, breaking the vitelline membrane and sub-blastodermal injection of Indian ink, required to visualize the embryo, lead to greatly decreased embryo viability and severe developmental defects. It is also extremely difficult to observe details of anatomical structures *in ovo* at these stages, particularly without ink or vital stains.

As young embryos cannot be electroporated *in ovo*, an *ex ovo* system is required. Many such methods have been described (reviewed in ref. 22), of which two are currently widely used: the ‘EC culture’ method<sup>17</sup>, in which the embryo is explanted attached to a piece of filter paper and subsequently transferred to an agar substrate on which it is cultured, and New culture<sup>18</sup> (modified as described in ref. 23), involving removal of the embryo attached to its own vitelline membrane, which is then stretched around a glass ring and cultured on a pool of egg albumen. Although EC cultures are slightly faster to set up than New cultures, EC-cultured embryos younger than about stage 3 develop well only in about half of the cases, and often have head defects (mainly microcephaly)<sup>17</sup>. These problems are not seen in New culture where survival is virtually 100% and which also allows the embryo to be detached from the membrane to access the epiblast from either side before replacing it on the membrane.

Both our method (based on modified New culture) and that used by the group of Weijer<sup>15,16</sup> (based on EC culture) target the epiblast from its apical side. Although explantation by the New culture method is slightly slower than by EC culture, the latter requires more time and care to detach the embryo from its

membrane to deliver the electroporation solution between the vitelline membrane and the apical side of the epiblast and it is much more difficult under these conditions to localize the injected DNA. We therefore chose the method described here (based on New culture) because electroporation can be controlled more precisely and because it allows a greater proportion of electroporated embryos to develop normally, thus offering greater reproducibility.

**Effects of saline composition.** For embryological experiments on early embryos, two salines are commonly used: Tyrode's and Pannett–Compton<sup>24</sup>. The former (which contains glucose and is buffered with bicarbonate) is usually chosen when embryos are collected in relatively large numbers, detached from their membrane and yolk, and then used for dissection or as donors for transplantation. The latter (which is phosphate buffered) is more commonly used for explantation of the embryo attached to its membrane for subsequent culture. We compared these two salines in terms of the efficiency of electroporation at different stages and found important differences at primitive streak stages. Plasmid DNA can be electroporated well in both salines (perhaps marginally better in Tyrode's) at all stages. On the other hand, MO cannot be electroporated well into the area pellucida (although they can into the area opaca) in Pannett–Compton saline. This becomes much less severe in Tyrode's and the problem is virtually eliminated by

inclusion of a small amount of plasmid DNA in the electroporation mixture. We also tested the effect of varying the ionic strength of Pannett–Compton saline while maintaining its osmolarity, but did not detect any effect.

**Morpholinos must be fluorescein labeled.** Fluorescein-labeled MO (obtained as such from GeneTools) can be electroporated efficiently through the method described here. These have the advantage that the electroporated cells can be visualized immediately after targeting (even using a dissection microscope equipped with fluorescence optics) and it also appears that fluorescein-coupling enhances the efficiency of electroporation, presumably because the fluorescein moiety is negatively charged. We have not tested whether lissamine- or biotin-labeled MO can be electroporated efficiently.

**Choice of electroporator.** Although it is commonly believed in the field that clean, square-wave pulses of voltage are required for successful electroporation, other colleagues claim that electroporators giving a less vertical ramp are more efficient and there is currently no consensus. The electroporating devices used in our laboratory (TSS10 model from Intracel and the more recent TSS20 'Ovodyne', with or without EP21 current amplifier) do not generate clean square waves (unlike the BTX 830) yet we have found the former to be very efficient and reproducible.

**MATERIALS**

**REAGENTS**

- Hens' eggs incubated 0–18 h (depending on stages needed)
- Sucrose (VWR, cat. no. 10274) 60% (wt/vol) in double-distilled water
- Fast Green FCF (Merck) 0.4% (wt/vol) in double-distilled water
- Pannett–Compton saline (see REAGENT SETUP)
- Tyrode's saline (see REAGENT SETUP)
- Experiment-specific plasmid DNAs and/or morpholinos (see REAGENT SETUP)

**EQUIPMENT**

- Dissecting microscope (with transmitted light base)
- Two pairs of watchmakers' forceps (number 4 or 5)
- A spoon/spatula (or small teaspoon)
- Pyrex baking dish ~5-cm deep, 2 l capacity
- 35-mm Plastic dishes with lids
- Watch glasses, ~5–7 cm diameter
- Rings cut from glass tubing, ~24–27 mm inner diameter, 1–2 mm thick and 3–4 mm high
- Plastic box with lid for incubating culture dishes
- 38 °C incubator
- Electroporator (EQUIPMENT SETUP)

**REAGENT SETUP**

**Pannett–Compton saline** Solution A: 121 g NaCl, 15.5 g KCl, 10.42 g CaCl<sub>2</sub> · 2H<sub>2</sub>O, 12.7 g MgCl<sub>2</sub> · 6H<sub>2</sub>O, H<sub>2</sub>O to 1 l. Solution B: 2.365 g Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O, 0.188 g NaH<sub>2</sub>PO<sub>4</sub> · 2H<sub>2</sub>O, H<sub>2</sub>O to 1 l. Both solutions can be autoclaved for storage at room temperature (18–23 °C). Once opened, the bottles can be kept at 4 °C. Before use, mix (in order): 120 ml solution A, 2,700 ml H<sub>2</sub>O and 180 ml solution B. If infections are a problem, antibiotic-antimycotic (GIBCO) solution (at 1:1,000 dilution) may be added.

**Tyrode's saline** A 10× concentrated stock can be made and autoclaved for storage: 80 g NaCl, 2 g KCl, 2.71 g CaCl<sub>2</sub> · 2H<sub>2</sub>O, 0.5 g NaH<sub>2</sub>PO<sub>4</sub> · 2H<sub>2</sub>O, 2 g MgCl<sub>2</sub> · 6H<sub>2</sub>O, 10 g glucose to 1 l. Before use, add 20 ml of this 10× stock to 180 ml H<sub>2</sub>O.

**Plasmid DNAs** Can be prepared using commercial kits (e.g., Qiagen). Dissolve the pure, salt-free DNA in Tris–EDTA (pH 8.0) to make a stock of at least 3 mg ml<sup>-1</sup>. **▲ CRITICAL** It is important to ensure that the stock solution contains no traces of alcohol, as this can damage the embryo or will lower the density of the electroporation mixture, making it difficult to apply.

**Morpholinos** Must be tagged with fluorescein for electroporation.

They can be designed and ordered from GeneTools. Their solubility varies; make a stock solution of at least 2 mM (we normally use 4 or 5 mM) in deionized sterile water.

**Electroporation mixture** Use water to dilute plasmids to a final concentration of 0.7–2.0 mg ml<sup>-1</sup> (according to their size) and/or morpholinos to 1 mM (we have used up to 3 mM morpholinos without noticing any toxicity, but 1 mM should suffice if one morpholino is to be used). Add 1/10 volume of 60% (wt/vol) sucrose and 1/10 volume of 0.4% (wt/vol) Fast Green FCF. Mix well by pipetting up and down gently with a Gilson. **▲ CRITICAL** We recommend making 5–10 µl of this mixture (or the amount required for one electroporation session) immediately before electroporation. This stock solution does not need to be kept on ice during the session. **▲ CRITICAL** Morpholinos alone electroporate very poorly into the area pellucida of embryos at primitive streak stages (HH3+ to 7). To electroporate morpholinos reliably into any cell population in the area pellucida of primitive-streak-stage embryos, we recommend adding a small amount of plasmid DNA to the electroporation mixture; a final concentration of 0.3 mg ml<sup>-1</sup>, or even severalfold less, works well.

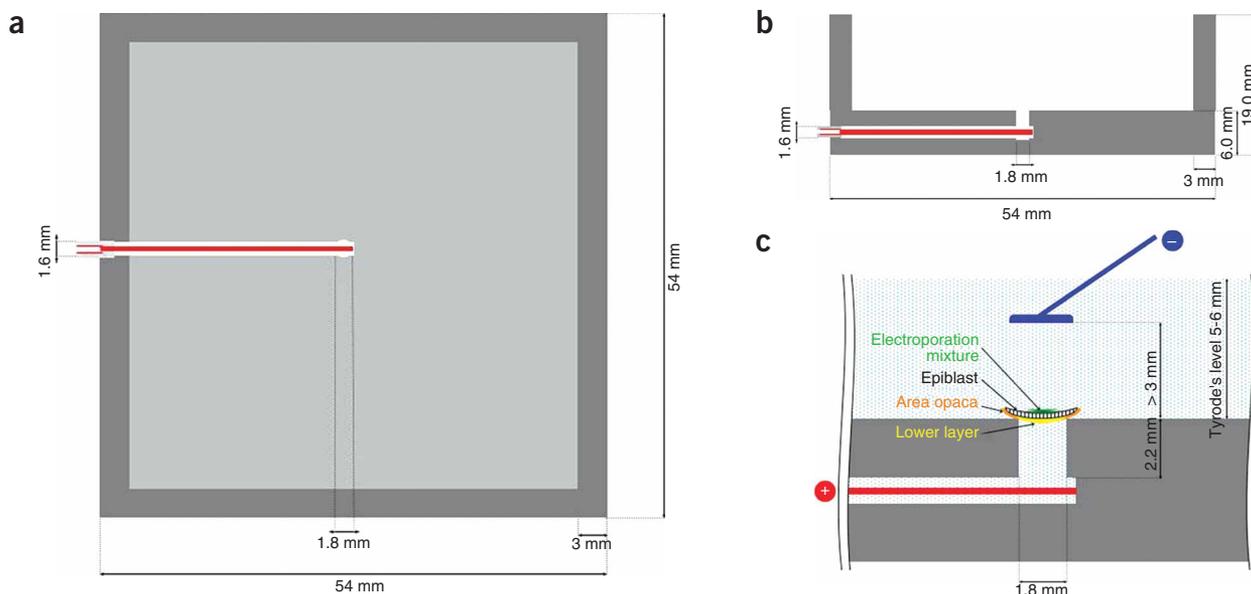
**EQUIPMENT SETUP**

**Electroporation chambers** The electroporation chamber will hold the positive electrode. It can be constructed from acrylic (Perspex) or similar plastic materials. A rectangular box (**Fig. 1**) can be made easily from sheets glued together. Before assembling (gluing) the chamber, drill a horizontal canal (~1.6 mm in diameter) through the center of the base sheet to just beyond the middle of the sheet, then drill a vertical hole, 1.8 mm diameter, through the middle of the sheet, just to meet the canal.

**Positive electrodes** Depending on the experiment, different sizes of electrode wire may be required.

To make a thin electrode (for small focal electroporation), solder one end of fine platinum wire (Goodfellow, cat. no. PT005127) to a mini-plug socket (SLS-1B, Multi-Contact, cat. no. 22.2603-22). Trim the other end of the wire so that, when the wire is inserted into the horizontal canal, its end runs through the length of the canal (through the central vertical hole). Insert the wire and seal the canal with water-resistant glue (e.g., Epoxy) just before push-fitting the conical socket. Very fine, pointed electrodes of Pt/Ir can also be ordered from FHC (5Exp/65mm Heat Shrink, cat. no. UEPM-GAVNNN2M).





**Figure 1** | Electroporation chamber. (a) Top view of the chamber. (b) Cross-section through the middle of the chamber, along the central canal (positive electrode). (c) Close-up of the electroporation setup, indicating the position of the embryo, application of electroperation mixture and the optimum distance of the negative electrode above the embryo.

A larger (plate) electrode can be made from silver wire (Goodfellow, cat. no. AG005150): roll one end of the wire into a spiral of 1.6 mm diameter, cover it lightly with silver soldering alloy (4% Ag, 95.5% Sn, 0.5% Cu) and polish it so that it can be slid inside the horizontal canal to reach into the central hole. As above, solder it to a mini-plug socket, seal the canal and push-fit the socket into the canal.

Solder a mini-plug (male) connector (SLS-1S; Multi-Contact, cat. no. 22.2602-22) to the end of a cable (50–100 cm standard insulated copper wire, red) and a red male banana plug to the other end. This red cable will be used to connect the electroperation chamber to the positive pole of the electroperator.

**Negative electrode** The negative electrode can be made from a short length of the same material as the positive ones (see above), soldered to insulated cable (50–100 cm standard insulated copper wire, black) with a black male banana plug soldered to the other end. This will be connected to the negative pole of the electroperator. Feed the electrode end of the wire through the stem of a 2-ml plastic pipette and attach this to a micromanipulator (any type, e.g., Prior).

Small negative electrodes can be made from platinum wire, flattened to make a paddle of  $1 \times 2 \text{ mm}^2$ —this, in combination with the thin electrode box, can be used to produce focal electroperations at primitive streak stages. An intermediate-sized negative electrode (e.g., for electroperating lines) can be made by bending the end of a 3 mm length of platinum wire and placing it parallel to the wire positive electrode. For electroperation of large regions, a large, flat electrode can be made in a similar way to the plate positive electrode: roll the end of a silver wire into a spiral ( $\sim 2.3 \text{ mm}$  diameter) and bring the stem to its center. Solder the base of the stalk with Ag alloy and flatten the underside (which will face the embryo during electroperation) by grinding it lightly with fine sandpaper.

**Electroperator** We have used both the older TSS10 model from Intracel and the more recent TSS20 ('Ovodyne', with or without EP21 current amplifier), with similar results. A foot switch is particularly useful. The BTX 830 square-wave electroperator is a suitable alternative.

## PROCEDURE

### Yolk collection ● TIMING 1–2 min per egg

1| Incubate eggs at  $38^\circ\text{C}$  to the required stage.

▲ **CRITICAL STEP** Morpholinos can be electroperated without the eggs having been preincubated; embryos withstand this very well. At pre-primitive streak stages, transfection of plasmid DNA requires that eggs be incubated at  $38^\circ\text{C}$  for at least 2 h before electroperation.

2| Break the shell by tapping the blunt end using coarse forceps, then open a round window, removing any sharp edges. See **Supplementary Video 1** online for a demonstration of Steps 2–5.

3| Collect the thin albumen into a 50-ml beaker by tipping the shell.

4| Discard the thick albumen, assisted with the coarse forceps.

5| Using the forceps, enlarge the window sufficiently to tip the yolk (still surrounded by its vitelline membrane) into the Pyrex dish filled with Pannett–Compton solution.

■ **PAUSE POINT** Yolks can be left in Pannett–Compton for up to 2 h before vitelline membranes are removed.

6| Repeat Steps 2–5 until enough yolks (5–15 is usually sufficient) have been collected.

## PROTOCOL

### Preparing vitelline membranes for New culture ● TIMING 2–5 min per egg

7| Lower a watch glass and a glass ring into the Pyrex container. See **Supplementary Video 2** online for a demonstration of Steps 7–12.

8| Turn the yolk using the side of the coarse forceps in a way that the blastoderm is uppermost (to the ‘North Pole’ of the yolk).

9| Using small scissors, make a cut just below the equator and continue to cut all the way around the yolk.

10| Using a pair of fine forceps, peel off the vitelline membrane from the North hemisphere (lying over the embryo) by pulling at the edges slowly but steadily. Primitive-streak-stage embryos tend to adhere to the membrane, whereas earlier embryos generally remain with the yolk.

11| Slide the membrane over the watch glass, with its inner (yolk side) surface upward. Place the glass ring over the membrane and pull at its edges in a manner that it protrudes from beneath the ring all around.

12| Remove this assembly from the saline and place it under the dissecting microscope. Add a small volume of Pannett–Compton solution inside the ring if all liquid has drained during transfer (never allow the embryo to dry out).

13| Working with fine forceps, fold the margins of the membrane over the edge of the glass ring; gently tug at the flaps to stretch the membrane around the glass ring uniformly but not too tightly. Trim any excess membrane inside the ring using scissors, working around its circumference. This process is shown in **Supplementary Video 3** online.

### Embryo dissection ● TIMING 1–3 min per embryo

14| Dissect the embryos and place them in a dish containing Tyrode’s saline. If the embryo remained in the yolk at Step 10, follow option A. If the embryo remained attached to the membrane at Step 10, follow option B.

#### (A) Dissecting embryos from yolk

(i) Use fine forceps to cut the yolk around and beneath the embryo. Steps 14A(i) and (ii) are shown in **Supplementary Video 4** online.

(ii) Use a spoon to pick up this plug and place it in a Petri dish containing Tyrode’s solution, with the embryo facing up.

(iii) Working under the microscope and using fine forceps, free the area opaca from the yolk carefully until the subgerminal cavity is reached. Fold the embryo, which facilitates freeing the rest of the area opaca from the yolk. For a demonstration, see **Supplementary Video 5** online.

(iv) Transfer the embryo to a small dish containing clean Tyrode’s.

■ **PAUSE POINT** Embryos can be kept in Tyrode’s for up to 3 h.

#### (B) Dissecting embryos attached to the membrane

(i) Work under the microscope and ensure that the glass ring is completely filled with saline. Steps 14B(i)–(iii) are demonstrated in **Supplementary Video 6** online.

(ii) Detach one corner of the embryo from the vitelline membrane using fine forceps. Work at a shallow angle and be careful to avoid damaging the vitelline membrane. Work around the edge of the embryo to free it completely from the membrane. It is also possible to free the embryo through gentle puffs of Tyrode’s saline from a Pasteur pipette, directed to the edge of the embryo.

(iii) Once the embryo has been freed, gently aspirate it into a large-mouthed pipette and transfer it to a small dish containing clean Tyrode’s.

■ **PAUSE POINT** Embryos can be kept in Tyrode’s for up to 3 h.

### Electroporation ● TIMING 1 min per embryo

15| Transfer each embryo to the electroporation chamber, filled with Tyrode’s solution, and arrange it to lie as flat as possible over the hole containing the positive electrode, with the region to be targeted slightly depressed. See **Supplementary Video 7** online for a demonstration of Steps 15–18.

▲ **CRITICAL STEP** In general, use Tyrode’s in the electroporation chamber, and add plasmid DNA to the electroporation mixture (see REAGENT SETUP): this is particularly important for driving morpholinos into the area pellucida of primitive-streak-stage embryos. To target morpholinos to the area opaca of embryos at primitive streak stages, use Pannett–Compton in the electroporation chamber.

16| Dispense the electroporation mixture over the intended area. A volume of <500 nl should suffice to cover the whole area over the hole.

▲ **CRITICAL STEP** Note that good spatial control can be achieved by applying the electroporation mixture carefully and quickly to the desired region. Success with the electroporation procedure is critically dependent on how well the electroporation mixture has

been applied over the desired area. To transfect large domains, the embryo should be placed flat over the hole and the mixture applied as uniformly as possible over the target area. For focal electroporations, the region of interest should be slightly depressed and the mixture applied locally. Arrange the embryo in the best configuration before dispensing the mixture, which will diffuse away quickly. Also, ensure that the negative electrode can be positioned rapidly and the pulses applied immediately after this. If the desired region bulges up, press it down very gently using the tip of a blunt forceps, manipulated at a shallow angle to the embryo. Do not use fine forceps or the capillary containing the electroporation mixture, which will puncture and damage the embryo.

**▲ CRITICAL STEP** We have noted that applying excess electroporation mixture appears to be harmful to pre-primitive streak embryos, and may cause the embryo to collapse soon after electroporation (within 2 h), whether they are kept at room temperature or placed in New culture. We recommend applying as small a volume of mixture as is practical.

**? TROUBLESHOOTING**

**17|** Position the negative electrode over this area quickly (at least 3 mm above the surface of the embryo) and apply current pulses, according to option A or B. Small bubbles will appear on the negative electrode.

**(A) Pre-primitive streak (EGK XI–XIV) and early streak stages (HH 2 and 3)**

(i) Apply five pulses of 4.0–4.2 V, 50 ms each, at 500 ms intervals. Use large plate electrodes, as smaller ones can result in damage to the embryo (presumably because of too high a current density).

**▲ CRITICAL STEP** Note that good spatial control can be achieved by applying the electroporation mixture carefully and quickly to the desired region. All cells in the epiblast appear to be equally prone to electroporation by DNA constructs, morpholinos or combinations of these.

**(B) Primitive streak stage embryos (HH 3+ to 7)**

(i) Apply three pulses of 5.6–6.0 V, 50 ms each, at 500 ms intervals. Any combination of electrodes can be used, depending on the intended electroporation pattern.

**▲ CRITICAL STEP** At these developmental stages, we observed regional differences in the efficiency of electroporation. Area pellucida epiblast cells, particularly in the prospective neural plate, are less prone to electroporation with morpholinos. The efficiency is greatly enhanced by the addition of plasmid DNA, even at low concentrations (see REAGENT SETUP). This DNA acts as a carrier and does not need to contain a promoter for transcription. It is also important to ensure that electroporation is carried out with the embryo immersed in Tyrode's saline rather than Pannett–Compton, which greatly reduces efficiency even further, for unknown reasons. Conversely, the area opaca epiblast can be electroporated readily with plasmid DNA, morpholinos or combinations thereof. If the area opaca epiblast is to be targeted specifically with morpholinos, we recommend using Pannett–Compton solution instead of Tyrode's in Step 14. In these conditions, very few cells in the area pellucida are electroporated, whereas the efficiency of morpholino electroporation in the area opaca is preserved; there is no need to add carrier DNA.

**18|** Remove the negative electrode and, using a glass pipette, gently blow over the surface of the embryo to remove excess electroporation mixture.

**19|** Transfer the electroporated embryo to a new dish containing Tyrode's and repeat Steps 14–18 for each embryo.

**■ PAUSE POINT** Embryos can be kept in Tyrode's for 1 h before being cultured.

**? TROUBLESHOOTING**

**Assembling the New cultures ● TIMING 2–4 min per embryo**

**20|** Pour 1.5–2 ml of thin albumen (collected in Step 3) into a 35-mm plastic Petri dish. See **Supplementary Video 8** online for a demonstration of Steps 20–26.

**21|** Aspirate each electroporated embryo from Step 19 into a wide-mouth pipette and place it over the vitelline membrane (prepared in Step 13), inside a glass ring.

**22|** Under the dissection microscope, turn the embryo in a way that its epiblast side (smooth side) faces the membrane. The orientation is most easily determined by observing the area opaca, whose ventral side (which should be facing upward) contains large yolk cells and therefore appears rougher than the epiblast side.

**23|** Working around the inside edge of the ring using a lightly flamed Pasteur pipette, aspirate the Tyrode's solution from inside the ring slowly, while ensuring the embryo remains in the center.

**24|** After all the saline has been removed from both inside and outside the ring, pick up the ring using coarse forceps and slide it away from the watch glass.

**25|** Place the ring over the pool of albumen in the 35-mm dish prepared in Step 20. Using two pairs of forceps, press down on the ring to prevent it from floating on the albumen. If there is a risk that the albumen might enter the ring, lower the level by removing some using a Pasteur pipette.



**26|** Pour some albumen onto the lid of the Petri dish and swirl it to distribute it uniformly, then pour the albumen back into the beaker. This will both seal the lid to the dish and prevent droplets of condensation from forming on the lid, which could damage the embryo. Place the albumen-coated lid on the dish and rotate the lid a full turn to seal.

**27|** When all cultures have been assembled, place them in a plastic box containing two paper towels soaked in water, close its lid and place it in an incubator at 38 °C for 3–24 h, as required by the experiment. Electroporation of fluorescently tagged morpholinos can be visualized immediately after electroporation, and they act very fast. Fluorescence of DsRed-Express or enhanced GFP from strong promoters in plasmid DNA can be observed after 3 h of culturing embryos; both the number of cells and the fluorescence intensity reach a plateau after 5–6 h. Electroporated cells can be visualized by direct fluorescence or by immunocytochemistry following fixation. Embryos electroporated and cultured through this method can be filmed directly, using either an upright or inverted microscope equipped with a thermoregulated hood. The culture dishes need to be sealed with Parafilm to prevent evaporation.

**? TROUBLESHOOTING**

**● TIMING**

Steps 2–5, yolk collection: 1–2 min per egg

Steps 7–13, preparing vitelline membranes for New culture: 2–5 min per egg

Step 14, embryo dissection: 1–3 min per embryo

Steps 15–19, electroporation: 1 min per embryo

Steps 20–26, assembling New cultures: 2–4 min per embryo

**? TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 1**.

**TABLE 1 |** Troubleshooting table.

Step	Problem	Possible reason	Solution
16	Morpholinos precipitate in the electroporation mixture	Morpholinos vary in their solubility, but most should stay in solution at 1 mM in the electroporation mixture for the 15 min or so required to electroporate 15–20 embryos (or more)	To avoid morpholinos precipitating, we recommend preparing the electroporation mixture just before electroporation and making just enough volume for one series of electroporations. Leftover morpholino can be kept at –20 °C and normally will not precipitate upon thawing
19	Embryos are scarred or ‘burned’ (electroporation site appears darker or blistered)	This may occur at primitive streak stages and can result from the negative electrode being too close to the embryo or excess DNA having been applied	Place the electrode at least 3 mm away from the embryo. At primitive streak stages, the volume of electroporation mixture delivered above the embryo appears to have little effect after formation of the primitive streak. Keep the concentration of DNA as low as possible while still giving good efficiency: usually, 1.0 mg ml <sup>-1</sup> is sufficient, and higher concentrations are needed only for electroporation of multiple DNA constructs and/or morpholinos
19 or 27	Embryos collapse after electroporation. This may happen for embryos electroporated with plasmid DNA at pre-primitive streak stages, in which case it can occur very soon after electroporation. Large holes appear in the epiblast, which then loses its integrity and collapses altogether	The negative (movable) electrode is too close to the embryo  The eggs have not been preincubated long enough  Too much DNA was applied to the embryo	Allow at least 3 mm (ideally 5 mm) between the electrode and the surface of the epiblast. Placing the electrode too close to the embryo may not only damage it, but is also ineffective  Determine the optimum preincubation period and stages at which the embryos can be safely electroporated (starting with stage EG XII, embryos should withstand the procedure very well). To this end, incubate eggs for various periods of time (at least 2 h at 38 °C)  Perform electroporations with an electroporation mixture containing plasmid DNA at a concentration of 0.5 mg ml <sup>-1</sup> . If the embryos still collapse or grow abnormally, the likely cause is that too much electroporation mixture was applied. Reduce the volume until the problem is overcome. Start by increasing the concentration of DNA in the electroporation mixture which results in good expression: in our experience, 0.5 mg ml <sup>-1</sup> should result in some cells being electroporated and expressing high levels of the reporter gene; increasing the concentration to 1.5–2.0 mg ml <sup>-1</sup> appears to give maximum efficiency



**TABLE 1** | Troubleshooting table (continued).

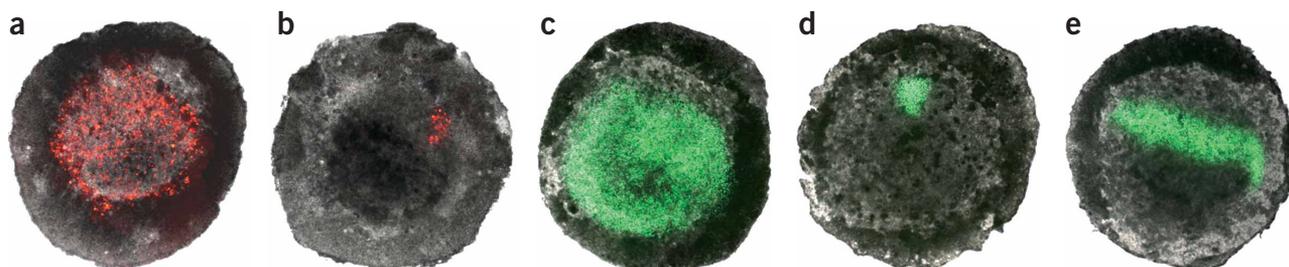
Step	Problem	Possible reason	Solution
27	Low efficiency of plasmid DNA electroporation		Make sure the DNA is pure (no contamination with bacterial chromosomal DNA) and that it contains a high proportion of the supercoiled form. Fresh preparations appear to work best. The efficiency of electroporation correlates better with the molar concentration of DNA than with its mass; therefore, comparable results should be obtained with equivalent molar concentrations. As a guideline, electroporation of 0.7 mg ml <sup>-1</sup> of 4 kb-long plasmids yields the highest efficiency; larger plasmids should be accordingly more concentrated to result in equivalent molar concentrations. We do not recommend concentrations > 2 mg ml <sup>-1</sup>
	Failure of plasmid DNA or morpholinos to electroporate		If the concentration of plasmid or morpholino is correct, check that small bubbles do form around the negative electrode during electroporation—these should be visible even when using the low-voltage protocol (4.2 V). If they do not, clean the electrodes and the dish: first, wash them with soapy water; then, fill the chamber with Tyrode's and apply 15 pulses of 10–12 V. Flush the electrodes with fresh Tyrode's, reverse the polarity and repeat the procedure. We do not recommend polishing the electrodes on a grinding stone or sandpaper. First, it reduces their lifespan; second, in the case of the plate electrodes used for early embryos, breaking the oxidation film presumably results in the current being too high and increases the incidence of embryo collapse

**ANTICIPATED RESULTS**

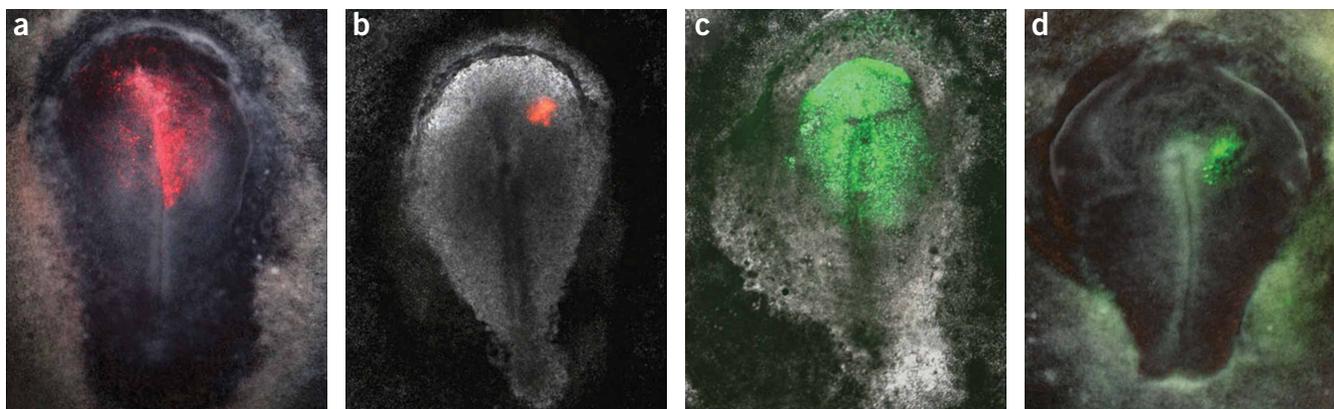
**Figures 2 and 3** show examples of electroporations of embryos before and after primitive streak formation, respectively. The red fluorescence is derived from a plasmid driving DsRed-Express from a chick cytomegalovirus promoter (pDsRed-Express-N1; Clontech), and green is fluorescein signal from a fluorescein isothiocyanate-labeled morpholino.

This type of experiment will be chosen mainly with the purpose of tracing cell fates and movements; for analysis of regulatory elements controlling gene expression; or for examining the effects of gain- or loss-of-function of one or more genes on the expression of other genes. For the former two, the embryos will usually be examined live, by imaging the fluorescence emitted by the fluorescent protein encoded in the plasmid and/or the fluorescein moiety attached to the morpholino. Conventional (epifluorescence), confocal or multiphoton microscopy may be used. It is also possible to fix the cultured embryos in 4% (wt/vol) formaldehyde and stain them with antibodies or subject them to *in situ* hybridization (either as whole mounts or as sections) for analysis of expression of marker genes, using standard techniques.

Morpholino electroporations are very efficient and, if the procedure is followed exactly, all embryos should grow normally. Electroporation of plasmid DNA is less efficient and can be harmful to preprimitive streak embryos. This critically depends on



**Figure 2** | Electroporation at pre-primitive streak stages. (a,b) Embryos electroporated with DsRed-Express. (c–e) Embryos electroporated with a control morpholino (coupled with fluorescein). All electroporations were carried out using flat, plate electrodes; localization was attained by applying the DNA/morpholinos locally.



**Figure 3** | Electroporations at primitive streak stages. (a,b) Large and focal electroporations of plasmid (DsRed-Express), respectively. (c,d) Large and focal electroporations of morpholino. The large electroporations were performed with flat, plate electrodes, focal ones with a small paddle.

the particular batch of embryos, and the minimum preincubation period has to be determined empirically. At least 80% of embryos should withstand electroporation well, and grow normally. As discussed above, targeting a precise area depends on several factors, and only one out of every two or three embryos may show perfect electroporation.

Note: Supplementary information is available via the HTML version of this article.

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**AUTHOR CONTRIBUTION** The first two authors contributed equally to this work.

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1. Stern, C.D. The chick; a great model system becomes even greater. *Dev. Cell* **8**, 9–17 (2005).
2. Muramatsu, T., Mizutani, Y., Ohmori, Y. & Okumura, J. Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos *in ovo*. *Biochem. Biophys. Res. Commun.* **230**, 376–380 (1997).
3. Nakamura, H., Katahira, T., Sato, T., Watanabe, Y. & Funahashi, J. Gain- and loss-of-function in chick embryos by electroporation. *Mech. Dev.* **121**, 1137–1143 (2004).
4. Chesnutt, C. & Niswander, L. Plasmid-based short-hairpin RNA interference in the chicken embryo. *Genesis* **39**, 73–78 (2004).
5. Das, R.M. *et al.* A robust system for RNA interference in the chicken using a modified microRNA operon. *Dev. Biol.* **294**, 554–563 (2006).
6. Katahira, T. & Nakamura, H. Gene silencing in chick embryos with a vector-based small interfering RNA system. *Dev. Growth Differ.* **45**, 361–367 (2003).
7. Hamburger, V. & Hamilton, H.L. A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92 (1951).
8. Sheng, G., dos Reis, M. & Stern, C.D. Churchill, a zinc finger transcriptional activator, regulates the transition between gastrulation and neurulation. *Cell* **115**, 603–613 (2003).
9. Uchikawa, M., Ishida, Y., Takemoto, T., Kamachi, Y. & Kondoh, H. Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals. *Dev. Cell* **4**, 509–519 (2003).
10. Kobayashi, D. *et al.* Early subdivisions in the neural plate define distinct competence for inductive signals. *Development* **129**, 83–93 (2002).

11. Uchikawa, M., Takemoto, T., Kamachi, Y. & Kondoh, H. Efficient identification of regulatory sequences in the chicken genome by a powerful combination of embryo electroporation and genome comparison. *Mech. Dev.* **121**, 1145–1158 (2004).
12. Iimura, T., Yang, X., Weijer, C.J. & Pourquie, O. Dual mode of paraxial mesoderm formation during chick gastrulation. *Proc. Natl. Acad. Sci. USA* **104**, 2744–2749 (2007).
13. Papanayotou, C. *et al.* A mechanism regulating the onset of Sox2 expression in the embryonic neural plate. *PLoS Biol.* **6**, e2 (2008).
14. Stern, C.D. & MacKenzie, D.O. Sodium transport and the control of epiblast polarity in the early chick embryo. *J. Embryol. Exp. Morphol.* **77**, 73–98 (1983).
15. Chuai, M. *et al.* Cell movement during chick primitive streak formation. *Dev. Biol.* **296**, 137–149 (2006).
16. Cui, C., Yang, X., Chuai, M., Glazier, J.A. & Weijer, C.J. Analysis of tissue flow patterns during primitive streak formation in the chick embryo. *Dev. Biol.* **284**, 37–47 (2005).
17. Chapman, S.C., Collignon, J., Schoenwolf, G.C. & Lumsden, A. Improved method for chick whole-embryo culture using a filter paper carrier. *Dev. Dyn* **220**, 284–289 (2001).
18. New, D.A.T. A new technique for the cultivation of the chick embryo *in vitro*. *J. Embryol. Exp. Morph.* **3**, 326–31 (1955).
19. Voiculescu, O., Bertocchini, F., Wolpert, L., Keller, R.E. & Stern, C.D. The amniote primitive streak is defined by epithelial cell intercalation before gastrulation. *Nature* **449**, 1049–1052 (2007).
20. Eyal-Giladi, H. & Kochav, S. From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Dev. Biol.* **49**, 321–337 (1976).
21. Takemoto, T., Uchikawa, M., Kamachi, Y. & Kondoh, H. Convergence of Wnt and FGF signals in the genesis of posterior neural plate through activation of the Sox2 enhancer N-1. *Development* **133**, 297–306 (2006).
22. Stern, C.D. & Bachvarova, R. Early chick embryos *in vitro*. *Int. J. Dev. Biol.* **41**, 379–387 (1997).
23. Stern, C.D. & Ireland, G.W. An integrated experimental study of endoderm formation in avian embryos. *Anat. Embryol. (Berl.)* **163**, 245–263 (1981).
24. Pannett, C.A. & Compton, A. The cultivation of tissues in saline embryonic juice. *Lancet* **206**, 381–384 (1924).



# CORRIGENDUM: Spatially and temporally controlled electroporation of early chick embryos

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In the version of this article initially published, the recipe for Tyrode's saline on p. 420, under Reagent Setup, contained two errors. "0.5 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ " should have read "0.5 g  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ". "2 mg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ " should have read "2 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ".

These errors have been corrected in the HTML and PDF versions of the article.