Transplantation of neural tissue: quail – chick chimeras

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Running title: Quail-chick chimeras

Summary

Tissue transplantation is an important approach in developmental neurobiology to determine cell fate, to uncover inductive interactions required for tissue specification and patterning as well as to establish tissue competence and commitment. Combined with state-of-the-art molecular approaches, transplantation assays have been instrumental for the discovery of gene regulatory networks controlling cell fate choices and how such networks change over time. Avian species are among the favourite model systems for these approaches because of their accessibility and relatively large size. Here we describe two culture techniques used to generate quail-chick chimeras at different embryonic stages and methods to distinguish graft and donor tissue.

1. Introduction

During development the entire central nervous system arises from the neural plate, which is induced in the ectoderm by signals from the organizer (*1-3*). Shortly thereafter, precursors for the forebrain, midbrain, hindbrain and spinal cord occupy different, albeit overlapping, territories (*4-7*). As the neural plate folds to form the neural tube, anterior-posterior and dorso-ventral patterning is established through signals from surrounding tissues, but also through the action of local organizers like the midbrain-hindbrain boundary, the floor plate and roof plate (*8*). Many of these paradigms were originally established by transplantation experiments using various ways to distinguish host and donor tissue. In particular, grafting experiments established fate maps of the neural plate and brain (*4, 5, 9-12*), the location and action of organizers (*8, 13*), and also the time of competence during which tissue can

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respond to organizer signals and the time when cells become committed to a particular fate (14-16). Although many of the signalling mechanisms that control these processes have been identified, the downstream transcriptional networks largely remain elusive. In this context transplantation experiments turned out to be critical tools to dissect inductive processes over time and to compare tissue interactions and organiser functions. For example, molecular screens allowed the discovery of new organisers in early development (17), while the comparison of different inductive processes revealed an unexpected similarity between the induction of the central and peripheral nervous system and how both diverge (18, 19). Thus, although transplantation assays are generally considered to be classical embryological approaches, combined with modern molecular techniques like transcriptional and epigenomic profiling they have the power to uncover gene regulatory networks that control cell fate choices.

Avian model systems have been very popular for such studies, because the development of their nervous system parallels that of mammalian embryos in many aspects and has been described in great detail. Unlike mammals, however, avian embryos are easily accessible, relatively cheap to obtain and little specialised equipment is needed for operations and for growing the embryos. Most, if not all transplantation experiments require a reliable system to distinguish host and donor tissue to locate the graft, to follow its progeny or examine cell behaviour like axonal projections, but also to establish whether e.g. changes in gene expression or neuronal morphology occur cell autonomously (e.g. within the graft) or are induced in surrounding cells (e.g. in neighbouring tissues). Many studies have used transplantation of tissues labelled with fluorescent dyes (e.g. (20), infected with retroviral vectors (transplanted into resistant hosts)(21) and more recently tissues from GFP-transgenic chickens (22, 23). However, one of the most extensively used techniques is cross-species transplantations generating chick-quail chimeras to provide permanent cell tracing (9-12). Quail and chick are closely related species; their early development is fairly similar,

but they differ very slightly in timing. The chimeras generated by transplantation of neural tissue and neural crest cells develop normally and are even able to hatch. Early experiments to distinguish quail and chick tissue made use of the fact that quail nucleoli are associated with a fair amount of heterochromatin, which is absent in most other species including the chick. Therefore, histological staining for DNA can differentiate quail and chick tissue (*24*). More recently, however, quail specific antibodies have become available, which either recognise all quail cells or quail neurites (*25, 26*). These are now frequently used and their detection can be combined with other techniques like in situ hybridization(*27*).

This chapter focuses on quail-chick transplantation of neural tissue at early neural plate and at later neural tube stages. After embryonic day 3-4, the brain becomes less accessible due to the formation of blood vessels and extraembryonic membranes, while the spinal cord remains accessible. The procedures described are used to replace neural tissue from chick with the identical tissue from quail (orthotopic) at the same stage of development (isochronic). However, similar strategies can be used for heterotopic or heterochronic grafts as well as to any other tissue.

2. Materials

All procedures described below require two pairs of watchmakers' forceps (number 5), one pair of coarse forceps, about 15 cm long, one pair of small, fine scissors, with straight blades about 2 cm long, Pasteur pipettes (short form), end lightly flamed to remove sharp edges and rubber teats, container for egg waste and small beakers (50-100 ml). Instruments should be cleaned with lightly soapy water, rinsed in distilled water and washed in 70% ethanol before drying on a tissue. You need a good stereo-microscope with transmitted light base and for *in ovo* work a cold light source (fibre optics) for illumination from the top. Fertile hens' or quails' eggs are incubated in a humidified incubator at 38°C until they have reached

the stage desired; staging of host and donor embryos is performed according to Hamburger and Hamilton (*28*). All solutions are diluted from autoclaved stock solutions in distilled water immediately before use; beakers for salines are autoclaved before use.

2.1 Materials for preparing chick hosts for New Culture

Operations on primitive streak to early somite stage (HH3$^+$-8) chick host embryos are performed in modified New culture (*29, 30*); at this stage embryos are fragile, difficult to manipulate *in ovo* and survival rate *in ovo* is poor. On the other hand, in New culture embryos can only be grown for 24-36 hrs even in an expert's hands. In addition to the above materials, this method requires a Pyrex baking dish about 5 cm deep with 2 liter capacity, watch glasses about 5-7 cm diameter, rings cut from glass tubing (approx. 27 mm outer diameter, 24 mm inner diameter and 3-4 mm deep; obtained from a local glass blower), 35 mm plastic dishes with lids (bacteriological grade) and a plastic box with lid for incubating culture dishes. Pannett-Compton saline is prepared from two stock solutions, which can be kept at 4°C if autoclaved; solution A: 121 g NaCl, 15.5 g KCl, 10.42 g CaCl$_2$·2H$_2$O, 12.7 g MgCl$_2$·6H$_2$O, H$_2$O to 1 liter and solution B: 2.365 g Na$_2$HPO$_4$·2H$_2$O, 0.188 g NaH$_2$PO$_4$·2H$_2$O, H$_2$O to 1 liter. To prepare working solution just before use, mix (in order) 120 ml A, 2700 ml H$_2$O and 180 ml B. Do not mix concentrated stocks of A and B.

2.2. Materials for preparing chick hosts for in ovo operation

After HH8-9, operations are performed *in ovo*; under perfect conditions embryos can be grown for a long time, even until hatching. Collect the following materials in addition to those listed at the beginning of the methods section. A scalpel with No. 3 handle and No. 11 blades, plasticine (Playdo) or foam (from packaging) to make a ring for resting eggs on their side, PVC tape to seal the eggs, 5 ml syringe with 21G needle (for removing albumen), 1 ml syringe with 27G (or finer) needle (for ink injection), 1 ml syringe with 21G needle (for

antibiotics), paper tissues, Indian ink (Pelikan Fount India or Windsor and Newton; diluted 1:10 in saline), silicone grease in a 10 ml syringe (no needle).

10x stock Tyrode's saline (80 g NaCl, 2 g KCl, 0.5 g NaH$_2$PO$_4$-2H$_2$O and 10 g glucose in 1 liter H$_2$O) is prepared in advance, autoclaved and kept at 4°C after opening. Dilute this to 1x working solution with autoclaved water (about 100ml are needed). 100x penicillin/streptomycin solution (Sigma A9909) and 70% ethanol are also required.

2.3. Materials for preparing quail donors

The following materials are required to harvest quail embryos: one glass Petri dish (10 or 15 cm diameter, depending on the number of embryos to be collected), one spoon-spatula for collecting embryos, a glass Pasteur pipette cut at the shoulder and fire polished (for embryo transfer), rubber teats, 500ml Tyrode's saline (see above), dissecting microscope with transmitted light base.

2.4. Materials for grafting

To dissect quail and chick tissue for grafting the following materials are required: 35mm Sylgard coated dish for dissecting (this should never come into contact with fixative), entomological insect pins (A1; steel) for pinning out embryos on the Sylgard dish, insect pins mounted on Pasteur pipettes or Tungsten needles (Goodfellow; 100µm diameter, mounted on aluminium holders or glass rods using sealing wax; sharpen by repeated exposure of the tip to a very hot Bunsen flame), 30G needles mounted on 1 ml syringes, P20 Gilson pipette and yellow tips. In addition, for *in ovo* transplantation in older embryos the following materials are needed: micro-knife (e.g. micro-feather microsurgery knives for eye surgery 15E blade angle), aspirator tube (Sigma A5177), 50 µl borsilicate glass capillaries (for trypsin injection) pulled to fine injection needles using an electrode puller, tips broken off (puller settings need to be determined; needles should be fine enough to avoid fluid uptake by

capillary forces, but large enough to deliver small amounts of trypsin by air pressure); about 50ml 0.12% trypsin (Difco) in Tyrode's saline, 5% serum (any species) in Tyrode's saline for stopping the Trypsin.

2.4. Materials for fixing embryos and analysing results

Embryos are fixed several hours or days after transplantation; they can be analysed by in situ hybridization or immunostaining to label specific tissues or cell types, followed by labelling with quail specific antibodies to detect the graft, by in situ hybridization using chick and quail specific probes or by histological sectioning followed by Feulgen and Rossenbeck staining to reveal nucleoli (*24*). Depending on the analysis, different fixatives are used.

Fixing requires a Petri dish for collecting embryos (for *in ovo*), 3.5 or 10 mm Sylgard dish for pinning out embryos, insect pins (see above), 7 ml glass vials and phosphate buffered saline (PBS). For whole mount in situ hybridization embryos are fixed in 4% PFA, 1mM EGTA in PBS, 0.1% Tween-20 and stored in methanol. For Feulgen staining and in situ hybridization on sections (*31*), embryos are fixed in Zenker's (50g $HgCL_2$, 25g $K_2Cr_2O_7$, 10g of Na_2SO_4 x10 H_2O in 1l distilled water; before use, add 5 ml glacial acetic acid to 100 ml of the solution) or Carnoy's fixative (50% ethanol, 11.1% formaldehyde, 10% glacial acetic acid), respectively. Further analysis requires materials for in situ hybridization; see detailed descriptions of these procedures in (*27, 31*).

The most important point for further analysis is the detection of quail tissue. While traditionally Feulgen staining has been used to reveal the difference between chick and quail nucleoli (*25, 32, 33*), now quail specific antibodies are the favorite method. The monoclonal mouse antibody QCPN (Developmental Studies Hybridoma Bank) labels all quail cells, while QN is specific for neurites (*26*). We generally perform QCPN staining after whole mount in situ hybridization; this procedure requires: glass vials, Pasteur pipettes and ruber teats, a

rocking platform, PBS, blocking buffer (1% goat serum, 0.5% Triton X100 in PBS), anti mouse IgG-HRP coupled (Jackson), 100mM Tris pH 7.4, 50mg/ml 3,3'-Diaminobenzidine (DAB) in 100mM Tris pH 7.4, 0.3% H_2O_2 in 100 mM Tris pH 7.4. It is also possible to perform in situ hybridization with a probe directed against sequences that differ between chick and quail (most likely the 3' UTR of a specific gene of interest) to distinguish transcripts produced by cells of the graft and donor (*34*).

3. Methods

3.1 Preparing quail donor embryos for grafting

Collect the materials listed under 2.3; to dissect the donor tissue, quail embryos are first removed from the egg and cleaned using the following steps.

1.   Remove quail eggs from incubator. Using fine scissors, gently tap the shell near the blunt end of the egg to penetrate the shell. Use the tip of the scissors to cut off a small cap of shell; avoid damaging the yolk.

2.   Pour thin egg white into waste; use the scissors to help and cut through the rather thick albumen if required (see Note 1).

3.   Once most albumen is removed, turn the yolk by stroking it very gently with the sides of the scissors to make the embryo become visible on top of the yolk.

4.   Using the scissors make 4 cuts into the vitelline membrane around the embryo; make sure that all the cuts meet (see Note 2).

5.   Using the spoon, pick up the square of embryo and membrane including a little yolk; try to collect as little yolk as possible.

6.   Transfer the embryo including yolk and membrane into the large Petri dish containing Tyrode's saline under a dissecting microscope by sliding it carefully off the spoon. Using fine forceps, turn the square of yolk/membrane/embryo so you can see the embryo.

7.   Once all donor embryos have been collected in the Petri dish, separate the embryos from adhering yolk. Work at low magnification; use two pairs of forceps to pick up a corner of the vitelline membrane with one and slowly but steadily fold it back, steadying the yolk with the other. Make sure that the membrane and embryo remain submerged in saline. The embryo will remain attached to the membrane. If not, peel off the membrane completely and then use the forceps gently to remove the embryo from the underlying yolk.

8.   Using the wide-mouth Pasteur pipette pick up the embryo, with (better) or without membrane, and transfer to a 10 cm dish with clean saline. To clean the embryo use a fire polished Pasteur pipette and gently blow saline over it; this will remove yolk particles. The embryos are now ready for dissection and grafting and can be kept for 1-2 hours before proceeding further.

3.2. Preparing chick hosts for New Culture

At primitive streak and neural plate stages, operations in avian embryos are most easily performed in New culture. The method described below is based on New's original technique (*30*) modified by Stern and Ireland (*29*). This modified culture method uses rings cut from glass tubes, instead of rings bent from glass rods and 35 mm plastic dishes instead of glass watch glasses resting inside a large glass Petri dish. The rings cut from tubing generate a slightly rough surface that grips the vitelline membrane and therefore allows

easy transfer of the culture into the plastic dish. Collect materials and solutions described in 2.1; to set up the cultures proceed as follows (Fig. 1):

1. Remove eggs from the incubator.

2. Fill the large Pyrex dish with about 1.5 l of Pannett-Compton saline; the volume should be large enough that eggs yolks are submerged completely.

3. To open an egg, tap its blunt egg with the coarse forceps and carefully remove pieces of the shell. Discard the thick albumen into the waste bucket, assisted with the coarse forceps. Collect the thin albumen in a small beaker (see Note 3).

4. Carefully tip the yolk into the Pyrex dish containing saline, taking care not to damage the membrane on the edges of the shell. Carefully turn the yolk with the side of the coarse forceps so the embryo is facing upwards. Now place a watch glass and a glass ring into the dish.

5. Cut the vitelline membrane enveloping the yolk just below the equator using small scissors; you can use one pair of forceps to push the yolk around gently, while continuing to cut all the way around its circumference (see note 4).

6. Using both pairs of fine forceps peel the vitelline membrane with the embryo attached slowly but very steadily off the yolk. Use one pair of forceps to pull the edge of the membrane slightly upwards (about 25-30° angle from the yolk surface) and the other to hold the yolk down. Do not pull tangentially along the yolk: this may detach the embryo from the membrane. Do not stop during this process. The embryo should come off with the membrane (see note 5).

7. Turn the vitelline membrane with the inner face containing the embryo pointing upwards and slide it, preserving its orientation, onto the watch glass. Place the ring over it so that membrane protrudes around the ring and the embryo sits in its

centre. Remove the watchglass, ring and embryo from the dish; tilt the assembly gently to pour off some saline while steadying the ring with one finger (see note 6).

8. Dry the bottom of the watch glass on some tissue. Using fine forceps, carefully wrap the lose edges of the vitelline membrane over the edge of the ring, all the way around its circumference. Pull the membrane slightly so its bottom is smooth and free from wrinkles, but be careful not to pull so tight that it breaks (see note 7).

9. Using the fire polished Pasteur pipette rinse the outside of the ring to remove yolk particles. If there is a lot of egg albumen remaining under the membrane, lift the ring gently and use the Pasteur pipette to remove it. Clean the yolk over and around the embryo using clean saline; be careful not to dislodge the embryo form the membrane. Damaged embryos do not grow well or normal. If there is a lot of vitelline membrane inside the ring, trim off the excess with the fine scissors while lifting the edges with fine forceps. At this stage embryos are ready for transplantation and can be kept on the bench for some time; make sure they remain well submerged under saline and there is sufficient saline on the watch glass. If keeping them for a few hours, place them on a wet tissue and cover with a large glass or plastic plate/dish.

10. To finalize the cultures after transplantations work under the microscope; carefully remove any remaining saline, both inside and outside the ring. Drying helps the graft and host tissue to heal faster. During culture the embryo and the inside of the ring must remain dry.

11. Pour some thin albumen (about 2-3 mm thick layer) on the bottom of a 35 mm Petri dish. Using fine forceps, slide the ring with vitelline membrane off the watch glass, and transfer it to the dish; lower it onto the albumen making sure that no air is trapped underneath. Press the ring lightly onto the bottom of the dish using two forceps to allow it to adhere.

12. Remove the excess albumen if its level comes close to the edge of the ring. At this point, remove any remaining liquid from inside the ring using a fire-polished Pasteur pipette. The vitelline membrane should be slightly dome-shaped; this will help to drain off any fluid that accumulates during culture (see note 8).

13. To seal the Petri dish, wet the inside of the lid with a thin film of albumen all around the egde, discard the excess and place onto the bottom part. Press lid down slightly to seal (see note 9).

14. Place the dish in a plastic box containing a piece of wet tissue, seal the box, and place it into an incubator at 38°C.

3.2. Preparing chick hosts for in ovo culture

Later stage embryos are generally grown *in ovo*, which allows embryos to grow for long periods, even until hatching. Eggs must be incubated lying on their side, so the yolk turns with the embryo facing upwards to make it accessible for manipulation. Collect the materials described in 2.2; to prepare hosts for *in ovo* operations use the following procedure (Fig. 2):

1. Remove the eggs from the incubator, place one egg onto the egg rest and clean with 70% ethanol; be careful not to rotate the egg.

2. Hold the 5 ml syringe with 21G needle nearly vertical and insert the needle into the blunt end of egg until you feel the shell at the bottom. Remove about 1 ml egg albumen and discard. This lowers the embryo, away from the top of the shell.

3. Using the scalpel score a 1 cm x 1 cm square on the top of the shell and lift it up using the blade or a pair of forceps.

4. Moisten the white membrane under the shell with a little Tyrode's saline and remove it with fine forceps, to the edge of the window. Be careful to avoid damage to the embryo underneath.

5.	Add saline to the egg so that the embryo floats up to the level of the window.

6.	Take up diluted ink into a 1 ml syringe with a 27G needle; make sure there are no air bubbles in the syringe. Insert the needle under the vitelline membrane almost parallel to the yolk surface; choose a position away from the embryo proper and point the needle towards and underneath the embryo. Inject about 50-100 µl ink; the amount should be kept as little as possible. Avoid moving the needle after initial penetration; otherwise the hole will become too big and yolk and/or ink will leak out. The embryo should now be clearly visible on a dark background (see note 10).

7.	Line the shell window with a shallow edge of Vaseline by ejecting it from the syringe. This will allow you to cover the embryo with a drop of saline during the operation for moisture and good optics. Fill the chamber with saline until there is a good dome of fluid. Adjust the fibre optic light so the light shines tangentially onto the embryo, which is now ready for manipulation (see note 11).

8.	Once the embryo has received the graft and it is in the correct position, remove the saline very carefully from above the embryo using a Pasteur pipette. Watch under low magnification to ensure the graft does not move. If required, reposition it using a pin mounted on a Pasteur pipette.

9.	Carefully insert the 5 ml syringe with 21G needle into the original hole in the blunt end of the shell. Carefully remove about 3 ml egg albumen to lower the embryo to its original position.

10.	Add 1-2 drops (50-100 µl) of antibiotic solution (see note 12).

11.	Use a tissue with 70% ethanol to wipe the Vaseline off the shell; dry the shell thoroughly with another tissue.

12.	Cut a piece of PVC tape about 3-4 cm long, stretch it slightly and allow it to relax again. To seal the egg, place the tape over the window and carefully smoothen any wrinkles without putting too much pressure on the shell. Make sure the edges of the

tape are firmly attached to the shell; if not they will roll up and expose the embryo.

13.   Incubate the egg in a well-humidified incubator at 38°C; next day you can turn the egg window side down which helps to keep the embryo moist and improves their development. Incubate for the desired period; generally the 2-4 day survival rate should be 80-90% (see note 13).

3.3. Grafting procedure: quail neural plate into chick hosts in New culture

The procedure below describes orthotopic, isochronic neural plate grafts from quail donors into chick hosts at $HH3^+/4$. The same method can be applied for heterotopic and heterochronic grafts or transplantation of other tissues.

1.   To prepare the host follow the procedure described under 3.1 until step 9.

2.   Replace one of the eyepieces of the microscope with an eyepiece containing a graticule; a protractor, to measure angles, is particularly useful.

3.   Place a host embryo from 3.1, step 9 (kept on a watch glass) under the microscope and center the graticule on the node.

4.   Define the area to be replaced using the graticule coordinates. New culture embryos face ventral side up; to reach the ectoderm lower layers need to be removed. Fold back the endoderm and mesoderm above the area to be replaced by quail tissue using 30G needles on a 1ml syringe serving as a holder. Use the sharp side of the needle gently to score both layers on three sides (e.g. anterior, posterior and lateral leaving them attached medially); then using the back of the needle carefully peel away the endoderm and mesoderm overlying the area to be grafted.

5.   Use tungsten needles, mounted insect pins or 30G needles to cut out the region of the neural plate to be replaced by quail tissue (see note 14; Fig. 1n). Set aside the host embryo and turn to the quail donor.

6.   Pin out a quail embryo of the same stage, ventral side up, on a Sylgard dish

containing Tyrode's saline; use insect pins through the extraembryonic region to stretch the embryo slightly.

7. Locate the area to be grafted using the graticule, remove the endoderm and mesoderm as described in step 4 and excise the underlying neural plate as described in step 5.

8. Working under low magnification, use a Gilson pipette to pick up the graft in 3-5 μl saline.

9. Move the host embryo back on the stage and, working under low magnification, place the graft close to the target site.

10. Use mounted insect pins to move the graft into the hole cut previously. It is crucial to maintain the apical-basal orientation of the graft; after excision the ectoderm generally contracts basally and the tissue curves slightly.

11. Carefully remove all the liquid outside the ring and most of the fluid inside the ring.

12. Flip back the mesoderm and endoderm to secure the graft in its position and carefully remove all remaining liquid inside the ring. Excess liquid around the grafted area should be removed using pulled capillaries on aspirator tubes (see note 15).

13. Now finish setting up the cultures by following steps 10-13 in section 3.2. Make sure that the dome of albumen is rather flat; a high dome causes too much tension and the grafts do not integrate properly. Leave embryos on the bench for 30-60 min to let the grafts heal and then proceed to step 14 in section 3.2.

3.4. Grafting procedures: quail neuroectoderm into chick host in ovo

This section describes orthotopic and isochronic neural tube grafts in embryos older than HH9; as with transplantations in younger embryos described above, the same techniques can be used for heterotopic and heterochronic experiments. First prepare the donor embryo and then turn to the host.

1. To prepare the quail donor, pin out the embryo dorsal side up on a Sylgard dish in Tyrode's saline and place under a dissecting microscope with transmitted light base.

2. Using tungsten needles or a mirco-knife, make a longitudinal incision into the ectoderm dorsal to the neural tube on both of its sides.

3. Replace the Tyrode's with trypsin solution; working at high magnification peel the ectoderm away from the neural tube using the back of a 30G needle. In the same way, gently scrape any loosely attached cells (neural crest depending on stage) off the neural tube and free it from the adjacent tissues (see note 16).

4. Progressively separate the neural tube from the underlying notochord using a microknife to push it from side to side, allowing the Trypsin to penetrate; when completely detached, cut the neural tube transversely at its anterior and posterior ends to free it.

5. Remove the excised neural tube using a using a Gilson pipette set to 3-5μl and place into a 35mm Petri dish containing saline with 5% serum (see note 17). Graft can be kept on ice until use.

6. Prepare the chick host by following steps 1-7 in section 3.2. Use a mounted insect pin make a small hole into the vitelline membrane just over the area to be operated. The hole should be as small as possible.

7. Replace the drop of Tyrode's saline with trypsin solution and follow steps 2-4 above to excise the same section of the neural tube as in the quail donor.

5. Remove the excised neural tube using a Gilson pipette set to 3-5μl and replace the trypsin solution with fresh Tyrode's saline twice.

13. Pick up the graft using a Gilson pipette, rinse in Tyrode's saline without serum before transferring it to the host.

14. Using a Gilson pipette and working under low magnification, transfer the graft into the saline bubble over the host embryo.

16. Use a mounted insect pin or 30G needle to place the graft into the hole made by removal of the host neural tube. Preserve anterior-posterior and dorso-ventral orientation (see note 18).

17. Once the transplant is in position, carefully remove the saline using a Pasteur pipette while observing under low magnification. If needed, re-position the graft using a mounted pin.

18. Finish the egg by following steps 8-13 in section 3.2.

3.5. Detecting quail tissue

As outlined above, grafted embryos can be analyzed in various ways depending on the question; these include whole mount or section in situ hybridization and tissue or cell specific immunohistochemistry. All of these techniques can be combined with the antibody staining using the quail specific antibody QCPN. We generally perform whole mount in situ hybridization for embryos up to embryonic day 3 and section in situ for older embryos followed by QCPN staining. The protocol below describes the whole mount procedure; for other applications see (*22, 23, 31*) . For embryos incubated to HH13 or older, it is a good idea to treat them with 6% H_2O_2 in PBS, 0.1% Tween after fixation or rehydration after storing in methanol (see: (*27*)); this reduces background for the in situ hybridization and immunostaining signal.

1. After developing the in situ hybridization color reaction, wash and fix embryos as normal. Remove fixative by washing in PBS three times for 10-30 min depending on the age of the embryo.

2. Block embryos for 1-3 hrs in blocking buffer at room temperature on a rocking platform.

3. Replace blocking solution with QCPN antibody solution (dilute antibody in blocking buffer; determine concentration for each batch of antibody) and incubate at 4°C on a rocking platform for two nights.

4. Remove antibody and wash embryos in PBS for 5-7 times one hour each; for older embryos leave the final wash overnight at 4°C.

5. Incubate embryos in secondary antibody (generally 1:1000 in blocking buffer, but may need titration) over one or two nights at 4°C.

6. Wash as in step 4.

7. Wash twice for 15 min in 100mM Tris pH 7.4 (see note 19); in the second wash measure the volume (generally 1ml is sufficient).

8. Add DAB from the stock to vial with embryos to a final concentration of 0.5mg/ml; incubate for 10-15 min rocking in the dark (see note 20).

9. Add the appropriate amount of H_2O_2 from the 0.3% stock to the embryos to make a final concentration of 0.003%.

10. Incubate in the dark until brown color develops normally within 5-10 min; check occasionally using illumination from the top on a white background.

11. Stop reaction by rinsing several times in distilled H_2O to remove residual substrate and post-fix embryos in 4% formaldehyde.

12. Embryos can now be cleared, photographed, embedded for paraffin or vibratome sectioning as required.

4. Notes

1. Remove as much albumen as possible; the yolks move less in the next step.

2. Make sure scissors are cleaned after each egg; crusts of egg yolk make the vitelline membrane stick to the scissors and the embryos tend to sink into the yolk. Do not hesitate when making the cuts, work rapidly so the embryos do not move.

3. Try to remove as much albumen as possible; albumen adhering to the vitelline membrane makes the following steps more difficult.

4. Make sure to cut at or slightly below the equator; otherwise it will be difficult to fit the membrane around the ring in the next step.

5. Occasionally embryos remain attached to the yolk particularly at early primitive streak stages. Keep the membrane because it can be used for other embryos, in case you accidentally punctured a membrane. The embryo can also be retrieved from the yolk, but requires thorough cleaning: use a pair of fine forceps, close them and gently push the edges of the extraembryonic region away from the yolk. Work all the way around the edge of the embryo. Transfer the embryo using a wide-mouthed pipette into a dish with fresh saline, ventral side up gently blow the attached yolk plug off the embryo using a Pasteur pipette. The embryo can now be returned to the membrane; make sure to keep it orientation: embryos do not grow with the ventral side on the membrane.

6. Make sure you do not turn the membrane inside out; embryos do not grow on the outer surface.

7. Be careful not to make any holes into the membrane; this will allow albumen to accumulate inside the ring and prevent the embryo from growing.

8. Be careful not to use too much albumen for grafted embryos; this will increase the tension and prevent healing.

9. Sealing is important to prevent condensation on the lid during incubation.

10. Too much or too high concentration of ink is toxic for the embryos. Recently Pelikan Indian ink has become difficult to obtain; if you are using other brands they need to be tested for toxicity.

11. A dome of liquid considerably improves the optics and also prevents the embryo from drying. The latter is critical as drying out reduces the survival rate.

12. Antibiotics are generally only required for long culture periods. Normally embryos survive well for 2-3 days without this.

13. Low survival rates can be due to a number of factors. The most common problems are dehydration, damage to critical blood vessels and infection. Working in a drop of saline helps to alleviate dehydration. To avoid infection ensure you use clean solutions; instruments can be cleaned periodically while working in distilled H_2O and ethanol (make sure it evaporates before using to operate), while tungsten needles are flamed periodically to keep them sharp.

14. Be careful not to make any holes in the vitelline membrane.

15. Drying the area surrounding the graft greatly improves healing; be careful not to suck up the graft into the capillary.

16. Be patient; the trypsin works almost by itself and it generally sufficient to push the adjacent tissues away using your instruments. Avoid going too deep and cut a hole into the endoderm; this will make ink and yolk leak out.

17. This inactivates trypsin; this is important because the tissue should be exposed to proteolytic enzymes for as short as possible to avoid disintegration.

18. If you encounter difficulties to preserve orientation, mark one end of the neural tube with a small crystal of carmine powder.

19. Make sure the pH is properly adjusted to 7.4; the reaction is pH sensitive.

20.   DAB is carcinogenic; make sure to wear appropriate protective clothing (lab coat,

gloves) and consult the local health and safety regulations for inactivation and

disposal.

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Figure legends

Figure 1. Setting up New culture. A. Set up. B. Instruments. C. Opening eggs. D. Removing albumen. E. Yolks in Pyrex dish. F. Cutting the vitelline membrane at the equator. G. Removing membrane and embryo from the yolk. H. Membrane with embryo facing upwards on watch glass. I. Placing glass ring on the membrane. J. Culture assembled on ring and watch glass. K. Removing assembly form Pyrex dish. L. Cleaning the culture. M. Primitive streak stage embryo on watch glass. N. Embryo after removal of a piece of neural plate. O. Setting up the culture in a Petri dish with albumen. P. Finished New culture.

Figure 2. In ovo culture. A. eggs are incubated on their side and placed on egg rest. Circle labels the blunt end of the egg. B. Blunt end is used to remove albumen with 5 ml syringe. C. Inserting syringe. D. Scoring the window. E. Windowed egg. F. Windowed egg with vaseline border surrounding the window. G. Embryo before ink injection. H. Ink injection using a 1 ml syringe. I. Ink injection: embryo is clearly visible. J. Embryo after ink injection. K. Removing albumen to lower the embryo. L. Eggs sealed with tape.
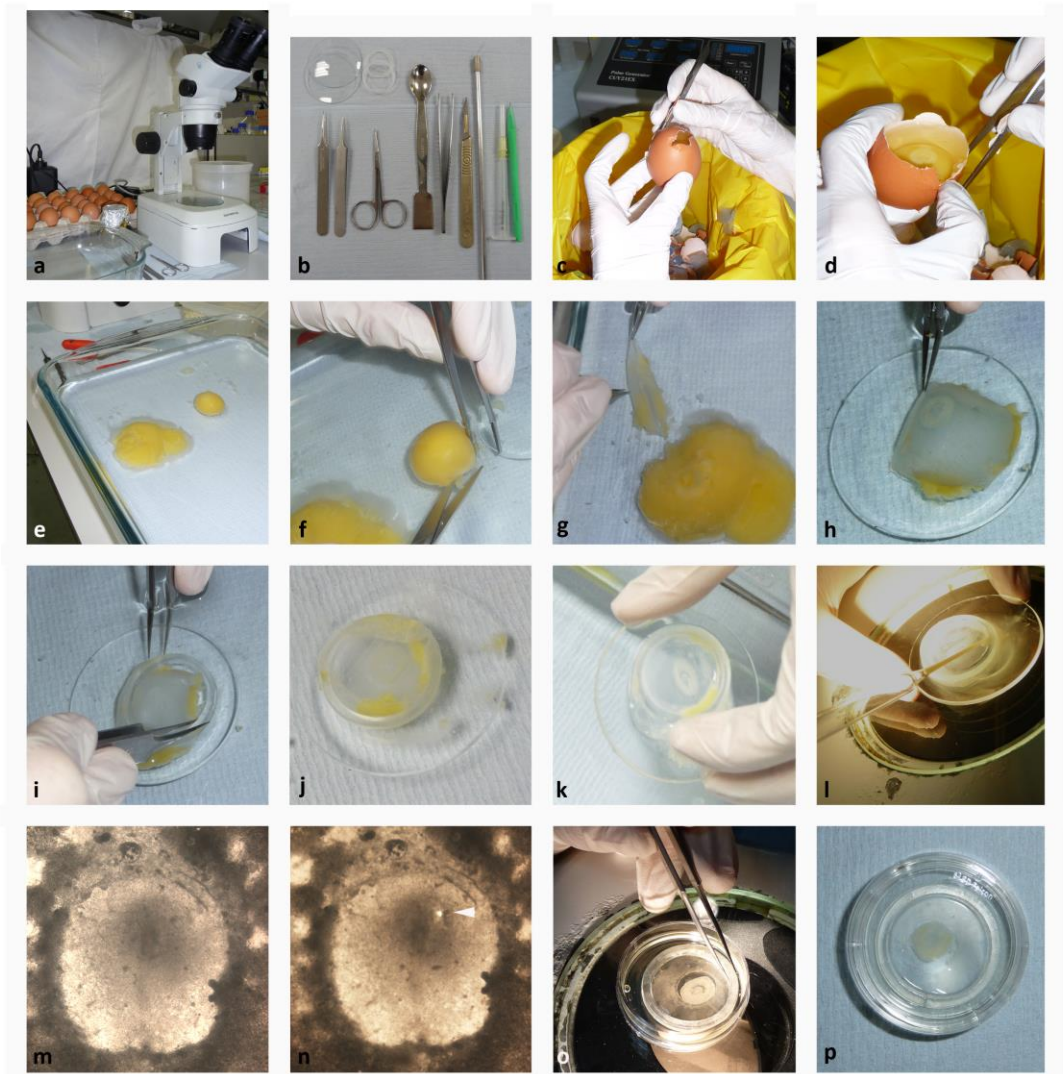
Figure 1:

Figure 2: