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Dr. Indrani Mukherjee
Science Editor of Journal of Visualized Experiments

Dear Dr. Indrani Musherjee,

We are submitting an article for publication in JoVE entitled “Isolation of embryonic tissues and formation of quail-chicken chimeric organs: the thymus example.” by Marta Figueiredo and Hélia Neves.

This manuscript provides a method to isolate pure embryonic tissues from quail and chicken embryos that can be combined to form *ex vivo* chimeric organs. This method was developed by Hélia Neves at N. Le Douarin’s Laboratory, INAF/CNRS, France and employed in Neves *et al.*, 2012[1] to study epithelial-mesenchymal interactions during early-stages of thymus formation.

Briefly, embryonic tissues are isolated by mechanical forces and subject to *in vitro* pancreatin digestion. In the described procedure, the conditions of enzymatic digestion, temperature and time of incubation, were optimized to preserve tissues biological properties.

With this method, the isolated tissues can be associated in an organotypic *in vitro* system for 48h. This system mimics the local cellular interactions in the embryo overcoming some restrictions of *in vivo* manipulation. Then, the capacity of the cultured tissues to form organs can be further tested using *in ovo* approaches as described in previous Jove’s publication[2].

The method presents an important tool for studying complex tissue interactions in developmental processes with highly dynamic spatial modifications and can also be applied to explore the potential/contribution of embryonic territories in organ formation.

Additionally, the isolation protocol allows three-dimensional preservation of the embryonic tissues particularly useful for detailing *in situ* gene-expression patterns of embryonic territories otherwise inaccessible by conventional methods. The transcriptome analysis approaches, including RNA-seq or microarrays, can also be applied in the isolated tissues without requiring genetic markers while providing a tissue-specific high throughput "omics" analysis.

We would like to acknowledge the kind invitation to publish this experimental procedure in JoVE and we hope that you will find it worth considering for publication.

With my best regards,
Sincerely,

Hélia Cristina Oliveira Neves

Hélia Neves
Assistant Professor, Medical School, University of Lisbon

[1]Neves, H., *et al*. N. M. Modulation of Bmp4 signalling in the epithelial-mesenchymal interactions that take place in early thymus and parathyroid development in avian embryos. *Dev Biol.* **361** (2), 208–219 (2012).
[2]Figueiredo, M. & Neves, H. Two-step Approach to Explore Early-and Late-stages of Organ Formation in the Avian Model: The Thymus and Parathyroid Glands Organogenesis Paradigm Video Link. *J. Vis. Exp* **5711437915** (13610) (2018).

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TITLE:

Isolation of Embryonic Tissues and Formation of Quail-Chicken Chimeric Organs Using The Thymus Example.

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KEYWORDS:

Embryonic tissue isolation; 3D-preserved tissues; *in vitro* organotypic assay; quail-chicken chimeric organ; thymus

SUMMARY:

This article provides a method to isolate pure embryonic tissues from quail and chicken embryos that can be combined to form ex vivo chimeric organs.

ABSTRACT:

The capacity to isolate embryonic tissues was an essential step for establishing the quail-chicken chimera system, which in turn has provided undisputed contributions to unveiling key processes in developmental biology.

Herein is described an optimized method to isolate embryonic tissues from quail and chickens by microsurgery and enzymatic digestion while preserving its biological properties. After isolation, tissues from both species are associated in an in vitro organotypic assay for 48 h. Quail and chicken tissues can be discriminated by distinct nuclear features and molecular markers allowing the study of the cellular cross-talk between heterospecific association of tissues. This approach is, therefore, a useful tool for studying complex tissue interactions in developmental processes with highly dynamic spatial modifications, such as those occurring during pharyngeal morphogenesis and the formation of the foregut endoderm-derived organs. This experimental approach was first developed to study the epithelial-mesenchymal interactions during early-stages of thymus formation. In this, the endoderm-derived prospective thymic rudiment and mesoderm-derived mesenchyme, were isolated from quail and chicken embryos, respectively.

The capacity of the associated tissues to generate organs can be further tested by grafting them onto the chorioallantoic membrane (CAM) of a chicken embryo. The CAM provides nutrients and

allows gas exchanges to the explanted tissues. After 10 days of in ovo development, the chimeric organs can be analyzed in the harvested explants by conventional morphological methods. This procedure also allows studying tissue-specific contributions during organ formation, from its initial development (in vitro development) to the final stages of organogenesis (in ovo development).

Finally, the improved isolation method also provides three-dimensionally (3D) preserved embryonic tissues, that can also be used for high-resolution topographical analysis of tissue-specific gene-expression patterns.

INTRODUCTION:

In the early 1970s, an elegant quail-chicken chimera system was developed by Le Douarin, opening new avenues to understand the role of cell migration and cellular interactions during development[1,2]. The model was devised on the premise that cell exchange between the two species would not significantly disturb embryogenesis, later confirmed when used to study numerous developmental processes, including the formation of the nervous and the hematopoietic systems[1]. Taking the latter as an example, the cyclic waves of hematopoietic progenitors colonizing the thymic epithelial rudiment was first observed using the quail-chicken chimera system[3]. For that, the prospective territory of the thymus, the endoderm of the third and fourth pharyngeal pouches (3/4PP), was mechanically and enzymatically isolated from quail (q) embryos at 15 to 30-somite stage [embryonic day (E) 1.5- E2.5]. These stages correspond to chicken Hamburger and Hamilton[4] (HH) - stages 12-17. The isolation procedures started with the use of trypsin to enzymatically dissociate the endoderm from the attached mesenchyme. The isolated endoderm was grafted into the somatopleura region of a chicken (c) host embryos E3-E3.5 (HH-stages 20-21). This heterologous mesenchyme was considered “permissive” to thymic epithelium development contributing also to the organ formation[3]. Afterward, successive waves of chicken host blood-borne progenitor cells infiltrated the quail donor thymic epithelial counterpart contributing to thymus formation in the host embryo[3].

More recently, a modified version of this approach was also proven to be important for studying epithelial-mesenchymal interaction during early-stages of thymus formation[5]. In this respect, the tissues involved in the formation of the ectopic thymus in chimeric embryos[3] were isolated, both from donor and host embryos, and associated ex vivo. An improved protocol was used to isolate the quail 3/4PP endoderm (E2.5-E3) and the chicken somatopleura mesoderm (E2.5-E3). Briefly, embryonic tissues were isolated by microsurgery and subject to in vitro pancreatin digestion. Also, the conditions of enzymatic digestion, temperature and time of incubation were optimized according to tissue-type and developmental stage (**Table 1**).

Next, the isolated tissues were associated in an organotypic in vitro system for 48 h, as previously reported[5,6]. The in vitro association of tissues mimics the local cellular interactions in the embryo, overcoming some restrictions of the in vivo manipulation. This system is particularly useful for study of the cellular interactions in complex morphogenic events, such as the development of the pharyngeal apparatus.

The contribution of each tissue in thymus histogenesis, as well as the ability of the heterospecific association to generate a thymus can be further explored using the CAM methodology, previously detailed[5,7,8]. Succinctly, the cultured tissues were grafted onto the CAM of cE8 embryo and allowed to develop in ovo for 10 days. Then, thymus formation was evaluated by morphological analysis in the harvested explants. As in the classical quail-chicken studies[3], the quail thymic epithelium was colonized by hematopoietic progenitor cells (HPCs) derived from the chicken embryo, which was later shown to contribute to organ development[9,10]. The HPCs migrated from the embryo to the ectopic chimeric thymus through the highly vascularized CAM[5,7,8]. Quail derived thymic epithelium can be identified by immunohistochemistry using species-specific antibodies (i.e., QCPN- MAb Quail PeriNuclear), overcoming the need for tissue-specific molecular markers.

This experimental method, as the two-step approach reported in previous publication[8], allows the modulation of signalling pathways by regular administration of pharmacological agents during in vitro and in ovo development. Also, explants can be harvested at any time-point of the course of the experiment[8].

Lastly, the isolation protocol here detailed allows the preservation of the natural properties and 3D-architecture of embryonic tissues, particularly useful for detailing in situ gene-expression patterns of embryonic territories otherwise inaccessible by conventional methods. In addition, transcriptome analysis approaches, including RNA-seq or microarrays, can also be applied in isolated tissues without requiring genetic markers while providing a tissue-specific high throughput "omics" analysis.

PROTOCOL:

All these experiments follow the animal care and ethical guidelines of the Centro Académico de Medicina de Lisboa.

1. Fertilized quail and chicken egg incubation

1.1) Place fertilized eggs of Japanese quail (*Coturnix coturnix japonica*) in a 38 °C humidified incubator for 3 days. Incubate the eggs (egg blunt end) facing up in the air chamber.

NOTE: The humidified environment is achieved by placing a water container at the bottom of the incubator.

1.2) Incubate fertilized eggs of chicken (*Gallus gallus*) for 2.5 days in a 38 °C humidified incubator. Incubate the eggs in a horizontal position and mark the upper side using a piece of charcoal to identify the embryo location.

NOTE: Start with 40 quail eggs and 60 chicken eggs when establishing this experiment.

2. Isolation of quail endoderm containing the prospective domain of the thymic rudiment

NOTE: Use a horizontal laminar flow hood and sterilized instruments and materials for egg manipulation procedures in sterile conditions.

2.1) Remove the embryonic region containing the presumptive territory of thymic rudiment, the pharyngeal arch region containing the 3rd and 4th arches (3/4PAR), as described[7,8].

2.1.1) Fill a large borosilicate glass bowl (100 mm x 50 mm; 100 cm^3) with 60 mL of cold phosphate-buffered saline solution (PBS).

2.1.2) With the help of curved scissors, tap and cut a circular hole in the shell of a quail egg that has been incubated for 3 days. Make the hole on the opposite side of the egg blunt and transfer the yolk (with the embryo) to the bowl with cold PBS.

2.1.3) Remove the embryo from the yolk by cutting the vitelline membrane externally to extra-embryonic vessels using curved scissors.

2.1.4) With the help of thin forceps, transfer the embryo to a small bowl (60 mm x 30 mm; 15 cm^3) filled with 10 mL of cold PBS.

2.1.5) With a skimmer, move the embryo to a 100 mm Petri dish with a black base (see **Table of Materials**) containing 10 mL of cold PBS and place it under a stereomicroscope.

2.1.6) Dissect the 3/4PAR, as previously described[8].

2.1.7) Aspirate the 3/4PAR and transfer to a glass dish three-quarters filled with cold PBS using a 2 mL sterile Pasteur pipette.

2.2) Isolate the endoderm containing the presumptive territory of thymic rudiment (the 3/4PP endoderm) by enzymatic digestion with pancreatin.

2.2.1) With the help of spatula and thin forceps, transfer the 3/4PAR to a glass dish three-quarters filled with cold pancreatin (8 mg/mL; 1:3 dilution of 25 mg/mL with cold PBS).

2.2.2) Incubate for 1 h on ice for enzymatic digestion.

NOTE: The time of enzymatic digestion depends of the stage of development (**Table 1**).

2.2.3) Place the glass dish under the stereomicroscope (40x–60x magnification) to isolate the endoderm from the 3/4PAR.

NOTE: Keep all surfaces and solutions cold during this procedure. Change to a new cold pancreatin solution if taking a long time to dissect the tissues (>15 min). As an illumination source,

use LED lights incorporated in the stereomicroscope or in the optic fibers, considering the limited heat load.

2.2.4) To isolate the endoderm from the surrounding tissues, use two stainless steel microscalpels in pin holders.

NOTE: Use microscalpels with a diameter between 0.1 mm and 0.2 mm and nickel pin holders with a jaw opening diameter of 0 mm to 1 mm.

2.2.4.1) First remove the neural tube and mesoderm attached to the dorsal surface of the pharyngeal endoderm.

2.2.4.2) With the dorsal side up, carefully detach and remove the mesenchyme between the pharyngeal arches and expose the pharyngeal pouches. Perform this procedure on both sides of the 3/4PAR.

2.2.4.3) Remove the heart tube and the mesenchyme surrounding the anterior pouches.

2.2.4.4) With the ventral side up, cut the ectoderm of the 2nd and 3rd pharyngeal arches and carefully remove the mesenchyme attached to the pouches. Repeat this procedure on the other side of the 3/4PAR. At this stage the thyroid rudiment should be visible.

2.2.4.5) Remove any remaining mesenchymal cells attached to the pharyngeal endoderm with the two microscalpels.

2.2.4.6) Make a transversal cut between the 2nd and 3rd PP, dissociating the pharyngeal endoderm containing the 3rd and 4th pouches from the anterior part of the endoderm having the thyroid rudiment and 2nd pharyngeal pouch.

2.2.4.7) With the help of spatula and thin forceps, transfer the isolated 3/4PP endoderm to a glass dish three-quarters filled with 100% cold fetal bovine serum (FBS).

2.3) Keep the glass dish with the isolated tissues on ice during the preparation of in vitro assay. Alternatively, the isolated tissues can be three-dimensionally preserved and in situ analyzed for gene-expression.

3. Isolation of chicken somatopleura mesoderm

NOTE: Perform egg manipulation procedures in sterile conditions using a horizontal laminar flow hood and sterilized instruments and materials.

3.1) Remove the embryonic territory containing the somatopleura mesoderm at the level of somites 19-24 (ss19-24).

3.1.1) Remove the chicken egg from the incubator after 2.5 days of incubation.

3.1.2) With curved scissors, open a small hole in the shell. Insert a needle and aspirate 2 mL of albumin with a 10 mL syringe to lower albumin volume inside the egg and prevent damage of the embryo (located below the marked region of the shell). Discard the aspirated albumin.

3.1.3) Cut a circular hole (up to two-thirds of the top surface area) in the marked region of the shell using curved scissors.

3.1.4) Cut the vitelline membrane externally to the extraembryonic vessels while holding the embryo with thin forceps.

3.1.5) Under a stereomicroscope, place the embryo in a 100 mm Petri dish with a black base containing 10 mL of cold PBS.

NOTE: Use a stereomicroscope from this point forward for progressive magnification of microsurgery procedures.

3.1.6) Use four thin insect pins to hold the embryo to the bottom of the plate. Place the pins in the extraembryonic region forming a square shape.

3.1.7) Perform two cuts between the somites 19 and 24 transversely to the embryo axis and crossing all embryo territory, using wecker eye scissors.

3.1.8) Release the embryo section, ss19-24, by cutting marginal embryonic edges.

3.1.9) Aspirate the ss19-24 tissues and transfer to a glass dish three-quarters filled with cold PBS using a 2 mL sterile Pasteur pipette.

3.2) Isolate the lateral mesoderm from somatopleura region (ss19-24) by enzymatic digestion with pancreatin (8 mg/mL; 1:3 dilution of 25 mg/mL with cold PBS).

3.2.1) With the help of spatula and thin forceps, transfer the ss19-24 tissues to a glass dish three-quarters filled with cold pancreatin solution.

3.2.2) Incubate for 30 min on ice for enzymatic digestion.

3.2.3) Under the stereomicroscope, isolate the mesoderm from the surrounding tissues using two microscalpels in a holder.

NOTE: Keep all surfaces and solutions cold during this procedure. Change to a new cold pancreatin solution if taking a long time to dissect the tissues (>10 min.). As an illumination source, use LED lights incorporated in the stereomicroscope or in the optic fibers, considering the limited heat load.

3.2.4) During mesoderm isolation, first remove the ectoderm at the surface followed by careful detachment of the ventrally located splancnopleura tissues.

3.2.5) Release the right lateral mesoderm of the somatopleura by cutting it in a parallel motion to the neural tube.

3.2.6) Repeat the mesoderm separation of the left side of the embryo.

NOTE: Make slow microscalpel movements during this procedure. The exposed extra-cellular matrix proteins stick to tissues and instruments preventing fluid movements.

3.2.7) With the help of spatula and thin forceps transfer the isolated mesoderm to a glass dish three-quarters filled with cold FBS.

3.3) Keep the glass dish with the isolated tissues on ice during the preparation of in vitro assay.

4. *In vitro* organotypic assay: heterospecific association of quail 3/4PP endoderm and chicken somatopleura mesoderm

4.1) Prepare the culture medium with RPMI-1640 medium supplemented with 10% FBS and 1% Pen/Strep[3,5].

4.2) Place a metal grid in a 35 mm Petri dish with 5 mL of culture medium.

NOTE: Remove the excess of liquid to level the medium surface with the top of the grid.

4.3) With the help of thin forceps, dip a membrane filter into the culture medium and then place it on the top of the grid to have one surface in contact with air.

NOTE: One-quarter of the membrane area (with 13 mm diameter) is adequate for the tissue association.

4.4) Under the stereomicroscope, associate the isolated tissues on the top of the membrane filter. First transfer the 3/4PP endoderm (step 2) from the glass dish by gentle sliding with the help of a transplantation spoon (or spatula) and thin forceps. Repeat this procedure for the isolated mesoderm (step 3).

NOTE: With the help of a microscalpel, mix the tissues to maximize its association.

4.5) Carefully place the associated tissues in a humidified incubator at 37 °C with 5% CO_2 for 48 h. Cultured tissues can be grafted onto the chorioallantoic membrane (CAM)**.**

NOTE: Ectopic organ formation in the CAM was previously detailed[8].

REPRESENTATIVE RESULTS:

The protocol details a method to isolate avian embryonic tissues to be used in several cellular and developmental biology technical approaches. This method was previously employed to study epithelial-mesenchymal interaction during early stages of thymus formation[5]. Herein, new results are shown in **Figure 1 and Figure 2**, using similar approaches.

[Place Figure 1 here]

Figure 1 is a schematic drawing of the endoderm isolated from the pharynx at qE3 (and cE3.5) (**Figure 1A**) and the in situ expression of two endoderm-related genes, *sonic hedgehog*[11,12] and *BMP7*[13] in the isolated tissue. The whole-mount in situ hybridization procedures were performed as previously described[5,7]. The expression of BMP7 was expressed in the endoderm of the 2PP and 3PP and excluded from the central pharynx and 4PP (probe was kindly provided by Elisabeth Dupin) (**Figure 1B**). Conversely, *sonic hedgehog* was detected in the endoderm of the central pharynx and excluded from the pouches[7] (**Figure 1C**).

[Place Figure 2 here]

Figure 2 depicts the experimental design used to develop ex vivo quail-chicken chimeric organs. The heterospecific association of tissues were grown in vitro for 48 h followed by in ovo development for 10 days (**Figure 2A**). Thymi formed in CAM-derived explants were identified by conventional histology. The thymus presented normal morphological features with well-developed medulla and cortex compartments (**Figure 2B,C**). Serial sections of the explants were further treated for immunocytochemistry (**Figure 2D-G**), as described[5,7]. The QCPN- MAb Quail perinuclear (**Figure 2D,E**) and anti-pan cytokeratin (CK) (**Figure 2F,G**) antibodies were used as markers for quail (species-specific) and epithelial cells, respectively. The chimeric thymus showed $QCPN^+$ thymic epithelial cells (**Figure 2D,E**), with reticular architecture (**Figure 2F,G**), and colonized by lymphoid cells (QCPN-) of donor origin (chicken).

FIGURE AND TABLE LEGENDS:

Figure 1. Representative results of gene-expression study of three-dimensionally preserved pharyngeal endoderm containing the presumptive territory of the thymus rudiment. Schematic representation of the pharyngeal apparatus and isolated endoderm containing the 2PP, 3PP and 4PP (at cE3.5 or qE3) (**A**). Whole-mount in situ hybridization with BMP7 (**B**) and Sonic Hedgehog (**C**) of isolated endoderm at cE3.5. Strong hybridization signals of BMP7 and Sonic Hedgehog pointed by white arrowheads in endoderm of the 2PP and 3PP (**B**) and central pharynx (**C**), respectively. A, anterior; cE, chicken embryonic day; D, dorsal; P, posterior; PP, pharyngeal pouch; qE, quail embryonic day; V, ventral. Scale bars, 50 µm.

Figure 2. Representative results of ex vivo formation of chimeric organs. Schematic representation of the experimental approach used to develop quail-chicken chimeric thymi (**A**). Briefly, the isolated quail 3/4PP endoderm (qE3) was associated in vitro with chicken

somatopleura mesoderm (cE2.5) for 48 h. The 48 h cultured tissues were then grafted onto the CAM (cE8) and allowed to develop in ovo for further 10 days. Serial sections of CAM-derived explants (**B-G**) were analyzed by conventional histology (**B** and **C**) and immunohistochemistry (**D** and **G**). In **B** and **C** (higher magnification of **B**), the slide was stained with H&E. In **D** and **E** (higher magnification of **D**) slide was immunodetected with QCPN antibody and counterstained with Gill's hematoxylin. In **F** and **G** (higher magnification of **F**) slide was immunodetected with anti-Pan CK antibody and counterstained with Gill's hematoxylin. Black arrow heads point to strong brown immunostaining of QCPN (**E**) and Pan CK (**G**). See **Table of Materials** for image acquisition details. Ca, cartilage; Ep, epithelium; PP, pharyngeal pouch; SoM, smooth muscle. Scale bars: 50 µm.

Table 1: Conditions of enzymatic digestion during embryonic tissues isolation.

DISCUSSION:

The embryonic tissue isolation procedure detailed here was improved from previous techniques to produce quail-chicken chimeric embryos in different biological contexts[3,5,6].

This approach is suitable to isolate pure embryonic tissues without requiring genetic manipulation or the use of tissue-specific markers, often limited in genetically modified animal models. It can be used to study epithelial-mesenchymal interactions during development, with the ability to isolate pure tissues being the limiting factor. For instance, as development progresses, tissues become thicker, more compact and attach to other neighboring tissues such that their separation is more difficult. This isolation procedure is, therefore, unsuitable for later stages of development, namely late-organogenesis.

This method is unique to study gene-expression in 3D-preserved embryonic tissues. To ensure the 3D-integrity of the isolated tissues, instruments, materials and solutions should be kept at low temperatures throughout the process.

The tissue microdissection procedure is also a critical step that relies, not only on the careful establishment of the experimental conditions (like temperature and duration of enzymatic digestion, as exemplified in **Table 1**), but also on the time-consuming hands-on training. This procedure requires patience and practice. If the operator loses the references of the region to be dissected, decreasing the stereoscope magnification (20x) will provide an overall observation that will help the next move decision.

The 48 h in vitro step was established to promote the cellular interactions between distinct embryonic tissues, while the in ovo tissue grown in the CAM supports the long-term development and chimeric organ formation of the heterospecific association of tissues[5]. The in vitro tissues associations may overcome some limitations of in vivo manipulations. For instance, local administration of drugs or growth-factors (using beads) in regions of the embryo otherwise inaccessible in vivo, can be easily performed using this in vitro approach. This has previously shown to mimic local tissue interactions during organ formation in the pharyngeal region[5].

Harvesting explants growing in CAM[5,7,8] is less time-consuming and is a simple method to track explants when compared to methods of collecting tissues grafted onto the body wall of chimeric embryos[3]. In addition, CAM can be transplanted with cells and tissues from other non-avian species, and it has been successfully used in several experimental contexts, from development to cancer[14,15]. For example, the CAM assay was previously applied in mice-into-chicken xenografts studies[16] and is frequently used to test the invasive capacity of human tumors cells[15].

Recently, an elegant study with human-into-chicken xenograft has validated the chicken embryo as a model to test and explore early human development[17]. In the future, it will be interesting to explore the methodology herein described using interspecies association of tissues, which may provide additional approaches to the mouse and human developmental studies.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

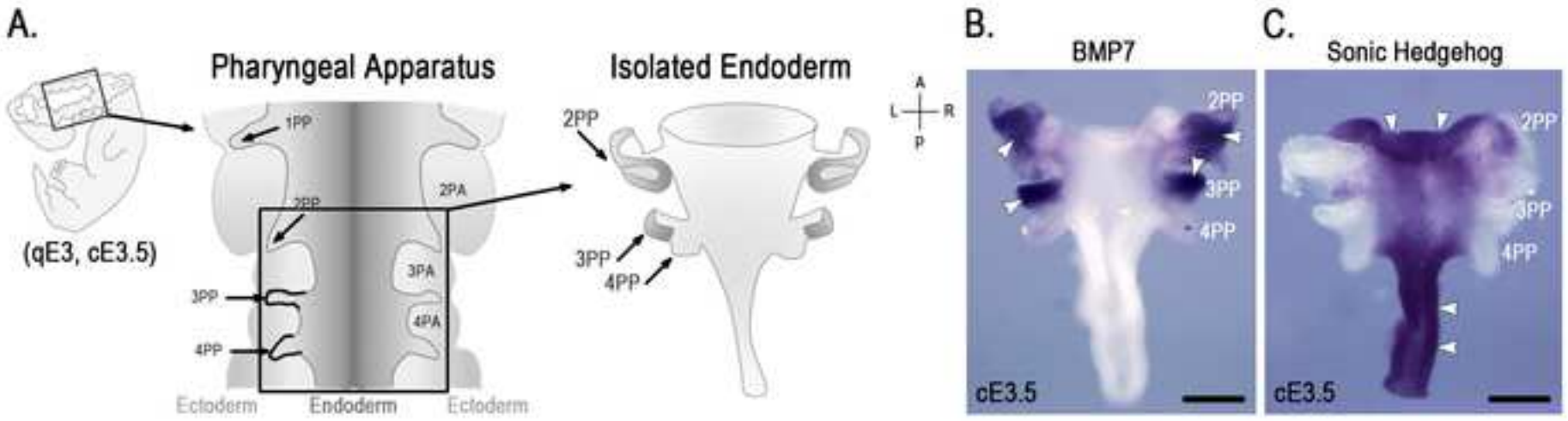

Figure 2

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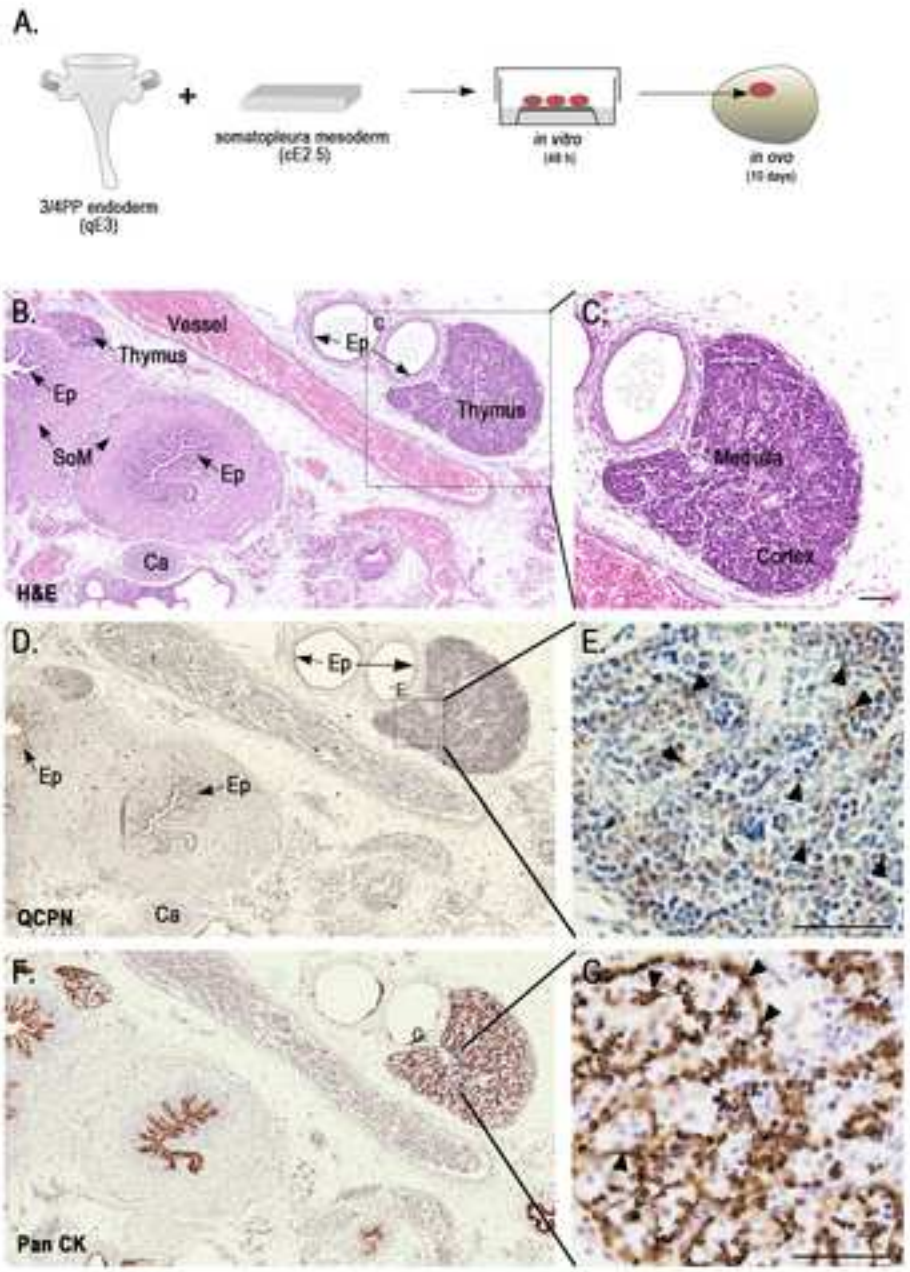

Table 1

Isolated tissue	Stage of development	Concentration
Pharynx endoderm	q E2 - E2.5 c E2.5 - E3	8 mg/mL
	q E3 c E3.5	8 mg/mL
	q E4 c E4.5	8 mg/mL
Somatopleura mesoderm	q E2 - E2.5 c E2.5 - E3	8 mg/mL

c, chicken; E, Embryonic day; q, quail.

Temperature	Incubation period
On ice	45 – 60 min.
On ice	60 - 90 min.
On ice	90 min.
On ice	30 – 50 min.

Table of Materials

Click here to access/download;Table of Materials;JoVE materials_Figueiredo&Neves.xlsx

Name of Material/ Equipment
Chicken fertilized eggs (*Gallus gallus*)
Quail fertilized eggs (*Coturnix coturnix*)
15 mL PP centrifuge tubes
50 mL PP centrifuge tubes
60 x 20 mm pyrex dishes
100 x 20 mm pyrex dishes
Metal grid
Membrane filter
Petri dish, 35 x 10 mm
60 x 30 mm pyrex bowls (Small size)
100 x 50 mm pyrex bowls (Large size)
Transfer pipettes
Glass pasteur pipette
Clear plastic tape
Cytokeratin (pan; acidic and basic, type I and II cytokeratins), clone Lu-5
Fetal Bovine Serum
Pancreatin
Paraformaldehyde
Penicillin-Streptomycin
Phosphate-Buffered Saline (PBS)
QCPN antibody
RPMI 1640 Medium, GlutaMAX Supplement
Bluesil RTV141A/B Silicone Elastomer 1.1Kg Kit
Dumont #5 Forceps
Extra fine Bonn scissors, curved
Insect pins
Micro spatula
Minutien Pins
Minutien Pins
Moria Nickel Plated Pin Holder
Moria Perforated Spoon
Wecker Eye Scissor
Camera
Microscope
NanoZoomer S360 Digital slide scanner
Stereoscope

Company	Catalog Number
Pintobar, Portugal	
Interaves, Portugal	
Corning	430052
Corning	430290
Duran group	21 755 41
Duran group	21 755 48
Goodfellows	
Millipore	DTTP01300
Sigma-Aldrich	P5112
Samco Scientific, Thermo Fisher Scientific	2041S
Normax	5426015
BMA Biomedicals	T-1302
Invitrogen, Thermo Fisher Scientific	
Sigma-Aldrich	P-3292
Sigma-Aldrich	P6148
Invitrogen, Thermo Fisher Scientific	15140-122
GIBCO, Thermo Fisher Scientific	10010023
Developmental Studies Hybridoma Bank	QCPN
GIBCO, Thermo Fisher Scientific	61870010
ELKEM/Silmid	RH141001KG
Fine Science Tools	11251-30
Fine Science Tools	14085-08
Fine Science Tools	26001-30
Fine Science Tools	10087-12
Fine Science Tools	26002-20
Fine Science Tools	26002-10
Fine Science Tools	26016-12
Fine Science Tools	10370-17
Fine Science Tools	15010-11
Leica Microsystems	MC170 HD
Leica Microsystems	DM2500
Hamamatsu Photonics	C13220-01
Leica Microsystems	Leica M80

Comments/Description
Poultry farm
Bird farm
fine meshed stainless steel grid
0.6 μm Isopore membrane filter
from supermarket
from supermarket
2 mL plastic pipet
from supermarket
Standart FBS
Prepare a 25 mg/mL solution according to manufacturer's instructions; centrifuge and filter prior to aliquote and store at -20ºC. Aliquots can be kept frozen for several years.
To prepare the back base for petri dish
Thin forceps
Curved scissors
0.3 mm Stainless steel pin
Transplantation spoon
0.2 mm Stainless steel microscalpel
0.1 mm Stainless steel microscalpel
Nickel plated pin holder
Skimmer

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Author(s): Marta Figueiredo and Hélia Neves

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Article Title:	Isolation of embryonic tissues and formation of quail-chicken chimeric organs: the thymus example.
Signature:	Hélia Cristina Oliveira Neves
Date:	18/08/2018

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10th November, 2018

Dr. Alisha DSouza

Senior Review Editor, JoVE

Dear Dr. DSouza,

Please find herewith the revised version of our manuscript entitled “Isolation of embryonic tissues and formation of quail-chicken chimeric organs: the thymus example” by M. Figueiredo and H. Neves.

We are thankful for the opportunity to resubmit a revised version of our manuscript and to reviewer’s insightful comments that helped us improve the manuscript.

Answers to the reviewer’s comments are provided in the accompanying file.

We hope you will find the changes satisfactory and the revised article suitable for publication.

Best regards,

Hélia Neves

Detailed answers (A) to reviewer's comments (R.C)

JoVE Scientific Review Editor

We are thankful to all comments and suggestions of the JoVE Scientific Review Editor that helped us improve the manuscript.

(1) Changes regarding the written manuscript:

R.C: "Significant portions show significant overlap with previously published work. Please re-write the text indicated in red in the attached document to avoid this overlap."

A: The text indicated in red was modified using track changes (see lines 104-137, 202-217, 265-271, 300-306 and 323-330).

R.C: "Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.."

A: In line 177, the sentence was re-written in the imperative tense, as requested, and the video narration modified accordingly.

R.C: "1.1: What is the relative humidity of the incubator? How is the humidity provided?"

A: An explanation to how humidity is provided was added in a new note in line 107.

R.C: "2.1.2: How large of a circle?."

A: Area size detail has been added in line 206.

R.C: "Please define the size of the Pyrex bowl. What is the volume?"

A: The size and volume of the two Pyrex bowl were added in lines 123 and 133, as requested.

R.C: "As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: a) Critical steps within the protocol; b) Any modifications and troubleshooting of the technique; c) Any limitations of the technique; d) The significance with respect to existing methods; e) Any future applications of the technique."

A: The discussion section was revised according to reviewer's suggestions. New paragraphs and sentences were added to cover the above-mentioned items:

- new sentence in paragraph 1, line 342 - Any limitations of the technique
- new paragraph 3 in line 350 - Critical steps within the protocol
- new sentence in paragraph 4, line 359 - The significance with respect to existing methods
- new sentence in paragraph 5, line 367 - The significance with respect to existing methods
- new paragraph 6 in line 372) - Any future applications of the technique

(2) Changes regarding the video:

R.C: "Please ensure that the additional details above are reflected in the video as well."

A: The video narration was changed according to text modifications.

R.C: "Please increase the homogeneity between the written protocol text and the video narration. This would help viewers to follow along the video with the text."

A: The video narration was been changed according to the written protocol text. The only exception is an additional video narration during live dissection of

tissues under the stereomicroscope aiming to strengthen a comprehensive approach to the surgical procedure and anatomic details (only observed in the video).

R.C: "**Branding concerns:** 2:44 - A "Pyrex bowl" is mentioned here. It doesn't seem like an intentional plug, but Pyrex is a brand name. They should probably say something like a "borosilicate glass bowl"."

A: The word Pyrex was removed from the written protocol text and audio narration. In line 123, "Pyrex" was replaced by "borosilicate glass" as suggested.

R.C: "**Audio issues:** The audio volume levels are a bit low and uneven in parts. All of the narration audio should be peaking between -12 and -6 dB."

A: The audio volume was levelled as requested.

R.C: "**Audio issues:** 3:50 - The audio becomes noticeably quieter here. This sentence should be rerecorded."

A: The pointed sentence was rerecorded.

R.C: "**Editing issues:** 2:50-3:28 - This sequence of actions is paced too quickly. The narration, especially, describes step after step with no pauses in between. This makes it more difficult for a viewer to clearly follow the action being presented. The pacing here should be slowed down."

A: This video section was modified with new video narration having pauses between distinct procedures.

R.C: "**Editing issues:** 3:16, 8:02 - The picture is fading to black while the narration is still describing a step. The fade down should happen after the narration is finished."

A: The fading to black was removed/shortened.

R.C: "Please ensure that the total video length remains below 15 min."

A: The total video length remains below the 15 min.

Comments from Peer-Reviewers:

We are thankful to reviewers #1, #2 and #3 for careful revision of the manuscript and suggestions that helped us to produce clearer messages.

Reviewer #1:

We are particularly appreciative to reviewer #1 for the careful revision of the manuscript and for highlighting the positive aspects of the work.

Minor Concerns

R.C: "It is not so clear to me that isolation of tissues was performed "by mechanical forces" as stated. It would be more appropriate to designate it as to "by microsurgery", since the tissue dissection procedure needs to be performed under a stereomicroscope and requires the use of microscalpels."

A: The expression "by mechanical forces" was replaced by "by microsurgery" in the introduction section of the text (see lines 25 and 69) and video (image and audio narration), as requested.

R.C: "Microscalpels and their preparation should be described more clearly in the text."

A: An additional note was added to the text detailing microscalpel preparation (see lines 161, 162). Microscalpel details were also added to table of materials.

R.C: "The authors could indicate which magnification of the stereomicroscope was used to perform endoderm and somatopleura isolation."

A: The magnification used to perform tissues isolation was added to the text protocol in line 154 (step2.2.3).

Reviewer #2:

Minor Concerns

R.C: "What is the difference between 'organotypic' and 'organoid'? Unless significant differences exist, perhaps the most widely used 'organoid' should be used."

A: An organotypic culture classically describes the in vitro interaction between two or more cell types (previously disaggregated), without three dimensions (3D) preservation, while an organoid is a miniaturization of an organ produced in vitro in 3D showing realistic micro-anatomy. Considering the significant differences between the two concepts and the procedures described, no changes were done regarding the term "organotypic".

R.C: "The enzymes used for enzymatic digestion and their concentration should be indicated in Table I. Incidentally, for how long can the 8mg/mL pancreatin solution kept frozen?"

A: Although pancreatin concentration is clearly indicated in the text and does not change in the different experimental conditions, a new column was added to table I with this information. Aliquots of 8 mg/mL pancreatin solution can be kept frozen for several years. This additional information is now provided in the table of materials.

R.C: "In the introduction, lines 73-74, the authors mention that 'the in vitro association of tissues … overcomes some restrictions of the in vivo manipulation". Can the authors elaborate on the in vivo manipulation in question and give examples of such restrictions?"

A: Considering the question raised by the reviewer, a new sentence was added in discussion section to exemplify such restrictions. "*For instance, local administration of drugs or grow-factors (using beads) in regions of the embryo otherwise inaccessible in vivo, can be easily performed using this in vitro approach, that has previously shown to mimic local tissue interactions during organ formation in the pharyngeal region*." (lines 360-363).

R.C: "Protocol, step 3.1.2. Can the authors explain either in the text or the video why should 2mL of albumin must be aspirated? Is the albumin kept?"

A: In the protocol text, an explanation was added about the need for albumin to be aspirated and discarded after aspiration (lines 203 and 204). An additional explanation, as to embryo location inside the chicken egg, was added to the sentences in lines 112 and 204.

R.C: "Protocol 3.2.6. Mesenchyme separation is indicated. Unless mesenchyme and mesoderm are equivalent tissues, this should be mesoderm separation. Description of this step in the video should also be modified."

A: According to the reviewer's recommendation, the word mesenchyme was replaced by mesoderm in the text (line 247) and in the corresponding audio narration.

R.C: "Protocol 4.3. Can the authors indicate whether the membrane filters should be cut before use? Pieces of membrane rather than full membranes appear to be used in the video."

A: Considering the question raised by the reviewer, an additional note was added to step 4.3 of the protocol to better describe membrane preparation (see lines 266 and 267).

R.C: "The induction of thymus organogenesis is presented as a representative result. Can the authors give examples of other tissue/organs that could potentially be induced and studied using this organoid culture system? Whether this organoid culture can be applied to other organism should be discussed. For instance, could chicken-human organoids be generated and cultured using this protocol? That would be useful for studies of, for example, cancer development and metastasis."

A: Considering the questions raised by the reviewer, two new sentences were added to the discussion section. "*In addition, CAM can be transplanted with cells and tissues from other non-avian species, and it has been successfully used in various experimental contexts, from development to cancer. For example, CAM assay was previously applied in mice-into-chicken xenografts studies and is frequently used to test the invasive capacity of human tumors cells*." (lines 367-370).

"*Recently, an elegant study with human-into-chicken xenograft has validated the chicken embryo as a model to test and explore early human development. In the future, it will be interesting to explore the methodology herein described using interspecies association of tissues, which may provide additional approaches to the mouse and human developmental studies*." (lines 372-375).

Reviewer #3:

Major Concerns

R.C: "Introduction, Line 59. Why was the somatic mesoderm used instead of splanchnic mesoderm or neural crests for quail-chick chimera system? The authors need to explain the reason."

A: The use of somatopleura mesoderm (and not other tissues) in this experimental approach is based in previous work (ref. 3), explained in the first paragraph of the introduction, line 58. *"The isolated endoderm was grafted into the somatopleura region of a chicken (c) host embryos E3-E3.5 (HH-stages 20-21). This heterologous mesenchyme was considered "permissive" to thymic epithelium development contributing also to the organ formation*[3]".

The adaptation in this experimental approach of the work referenced (in 3) is detailed in the second paragraph of the introduction, line 64. *"More recently, a modified version of this approach …. In this respect, the tissues involved in the formation of the ectopic thymus in chimeric embryos*[3] *were isolated, both from donor and host embryos, and associated ex vivo. An improved protocol was used …"*

R.C: "Protocol, (2.2.4) Line 160, (2.2.5) Line 176, (3.2.3) Line 234. What kind of material is the microscalpels? (tungusten needle, or pin?)."

A: Considering the question raised by the reviewer, details of microscalpel composition were added to the text in step 2.2.4 (see lines 161, 162). Other microscalpel details were also added to the table of materials.

R.C: "Protocol, (2.2.4.7) Line 183. What is the concentration of FBS, 100%."

A: The information of FBS concentration was added to the protocol text (line 185).

R.C: "Protocol, (4.2) Line 260. What kind of material is the metal grid made of?"

A: The composition of the metal grid is stainless steel. This information was added to the table of materials.

R.C: "Protocol, (4.4) Line 266. The isolated endoderm is Quail?"

A: The isolated endoderm is quail origin, as clearly stated in step 2 of the protocol. This information was further reinforced in the protocol, in line 270 of step 4.4.

R.C: "Protocol, (4.4) Line 268. The isolated mesoderm is Chick?"

A: The isolated mesoderm is chicken origin, as clearly stated in step 3 of the protocol. This information was further reinforced in the protocol, in line 272 of step 4.4.

R.C: "Protocol, (4.6) Line 274. Did the cultured tissues adhere to the membrane filter after incubation for 48 hrs? Is it easy to graft the tissues onto the CAM? The authors need to explain how to graft the tissues onto the CAM."

A: The details of CAM grafting procedures were detailed in previous Jove's publication, as indicated in the text of the protocol and video.

R.C: "Figure legends, Figure 2, Line 314-325. What do the arrowheads mean?"

A: The following sentence of the text clearly described the meaning of the arrowheads in figure 2 - "*Black arrow heads indicate QCPN (E) and Pan CK (G) strong immunostaining*." However, a modified sentence was written according to JoVE Scientific Review Editor's request. The new sentence is "*Black arrow heads point to strong brown immunostaining of QCPN (E) and Pan CK (G)*" (see lines 328 and 329).

R.C: "Figure 2 legend, Line 321. What color does the immunostaining for positive cells (brown color)? As it is difficult to distinguish the positive and negative cells in Fig 2E and 2G, the authors need to show higher magnification of the pictures."

A: The colour description (brown) of positive immunostained cells was added to the fig. 2 legend (line 329). As requested by the reviewer, the images in Fig. 2E and 2G were replaced with higher magnifications. The video was changed accordingly.