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.