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TITLE:

Chicken Recombinant Limbs Assay to Understand Morphogenesis, Patterning, and Early Steps in Cell Differentiation

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SUMMARY:

Recombinant limbs are a powerful experimental model that allows for studying the process of cell differentiation and the generation of patterns under the influence of embryonic signals. This protocol presents a detailed method for generating recombinant limbs with chicken limb-mesodermal cells, adaptable to other cell types obtained from different organisms.

ABSTRACT:

Cell differentiation is the fine-tuned process of cell commitment leading to the formation of different specialized cell types during the establishment of developing tissues and organs. This process is actively maintained in adulthood. Cell differentiation is an ongoing process during the development and homeostasis of organs. Understanding the early steps of cell differentiation is essential to know other complex processes such as morphogenesis. Thus, recombinant chicken limbs are an experimental model that allows the study of cell differentiation and pattern generation under embryonic patterning signals. This experimental model imitates an *in vivo* environment; it assembles reaggregated cells into an ectodermal cover obtained from an early limb bud. Later, ectoderms are transferred and implanted in a chick embryo receptor to allow its development. This assay was mainly used to evaluate mesodermal limb bud cells; however, it can be applied to other stem or progenitor cells from other organisms.

INTRODUCTION:

The vertebrate limb is a formidable model for studying cell differentiation, cell proliferation, cell death, pattern formation, and morphogenesis[1,2]. During development, limbs emerge as bulges

from the cells derived from lateral plate mesoderm[1]. Limb buds consist of a central core of mesodermal cells covered by an ectodermal coat. From this early structure, a whole and well-formed limb emerge. After the limb bud arises, three axes are recognized: (1) the proximo-distal axis ([PD] shoulder to fingers), (2) the dorso-ventral axis ([DV] from the back of the hand to palm), and (3) the anterior-posterior ([AP] thumb to finger). The proximal-distal axis depends on the apical ectodermal ridge (AER), specialized ectoderm located at the distal tip of the limb bud. The AER is required for outgrowth, survival maintenance, proliferation, and the undifferentiated state of cells receiving signals[2,3]. On the other hand, the zone of polarizing activity (ZPA) controls anteroposterior patterning[4], while the dorsal and ectoderm controls dorsoventral patterning[7,8]. Integration of three-dimensional patterning implies complex crosstalk between these three axes[5]. Despite understanding the molecular pathway during limb development, open questions about the mechanisms that control patterning and proper outgrowth to form a whole limb remain unanswered.

Edgar Zwilling developed the recombinant limb (RL) system in 1964 to study the interactions between limb mesenchymal cells and the ectoderm in developing limbs[6]. The RL system assembles the dissociated–reaggregated limb bud mesoderm into the embryonic limb ectoderm to graft it into the dorsal part of a donor chick embryo. The signals provided by the ectoderm induce the expression of differentiation genes and patterning genes in a spatio-temporal manner, thus inducing the formation of a limb-like structure that can recapitulate the cell programs that occur during limb development[7–9].

The RL model is valuable for understanding the properties of limb components and the interaction between mesodermal and ectodermal cells[6]. An RL can be defined as a limb-like structure created by the experimentally assembling or recombining limb bud mesodermal cells inside an ectodermal jacket[6]. The morphogenesis of the RL depends on the characteristics of the mesodermal cells (or other types) that will respond to the ectodermal patterning signals. One of the advantages of this experimental system is its versatility. This characteristic permits the creation of multiple combinations by varying the source of mesodermal cells, such as cells from different developmental stages, from different positions along the limb, or whole (undissociated) or reaggregated cells[7,10]. Another example is the capability of obtaining the embryonic ectoderm from species other than chicken, for example, turtle[11], quail, or mouse[12].

In this sense, the RL technique helps study limb development and the interactions between limb mesenchymal and ectodermal cells from an evolutionary point of view. This technique also has great potential for analyzing the capability of different sources of progenitor cells to differentiate into a limb-like structure by taking advantage of the signals provided by the embryonic ectoderm[12–14]. In contrast to *in vitro* cultures, the RL permits evaluating the differentiation and morphogenetic potential of a cell population by interpreting embryonic signals from a developing limb[9,15].

In this protocol, a step-by-step guide to performing successful RL with reaggregated mesodermal limb bud cells is provided, thus opening the possibility of adapting this protocol with different sources of reaggregated cells or even different ectoderm sources.

PROTOCOL:

This research was reviewed and approved by the Institutional Review Board for the Care and Use of Laboratory Animals of the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM, Mexico City, Mexico). A schematic flowchart of the general steps of this protocol is shown in **Figure 1A**.

1. Embryo incubation and determination of viability

1.1. Incubate fertilized chicken eggs at 38 °C and 60% relative humidity for about three and a half days until they reach the 22 HH stage (according to Hamilton and Hamburger, 1951)[16].

NOTE: Freshly fertilized chicken eggs can be stored at 15 °C for up to one week.

1.1.1. To incubate the fertilized eggs, place the eggs vertically with the pointy side down into the humidified incubator. Rotate the eggs during incubation as it is necessary to prevent the developing embryo from adhering to the shell membrane.

NOTE: Fertilized hen eggs can be obtained from local farms. Fertilized White Leghorn chicken eggs are generally used to avoid pigmentation of the feathers in late embryos. Consider incubating enough eggs separately to obtain three structures: (1) limb mesodermal cells, (2) limb ectoderms, and (3) donor embryos to graft the RL.

1.2. After three and a half days of incubation, remove eggs from the incubator, swab with 70% ethanol, and allow to air dry.

1.3. Identify developing embryos candling the egg to observe the blood vessels and locate the embryo. Discard eggs that do not have an embryo.

NOTE: At this point, distribute the eggs to obtain mesodermal cells, ectoderms, and the hosts for the RL.

2. Obtaining limb mesodermal cells to fill ectoderms

NOTE: Before initiating the manipulations, it is highly recommended to disinfect the working area, the microscopes, and all the instrumental by swabbing with 70% ethanol solution.

2.1. Tap the blunt end of the eggshell with the end of a blunt forceps to open a window and remove about 1 cm x 1 cm of the shell using the forceps.

2.2. Transfer eggs to a plastic or carton holder and placed them one by one under the stereomicroscope. Identify the air membrane and then remove it by picking a small hole where no vessels are found. Pull this area with the aid of fine surgical forceps.

NOTE: The air membrane can be identified as the white and opaque membrane observed immediately after windowing the egg.

2.2.1. Remove any small piece of eggshell that may come into contact with the embryo.

NOTE: Verify that the embryos are in the 22 HH stage before initiating the protocol. Embryos in earlier stages can be returned to the incubator after sealing the eggshell window with tape.

2.3. Open the amniotic sac completely by tearing open the amnion using the fine surgical forceps (see **Table of Materials**).

NOTE: Amnion can be identified as a transparent membrane that closely covers the embryo and is filled with amniotic fluid.

2.4. Carefully remove the embryo from the egg using a pair of blunt forceps and then transfer into a sterile Petri dish containing ice-cold phosphate-buffered saline solution (1x PBS). Repeat this step for the remaining eggs. Consider that only ~8-10 embryos are sufficient to obtain the mesodermal cells.

2.5. Wash the embryos once in ice-cold 1x PBS, and with the aid of a stereomicroscope (see **Table of Materials**), withdraw any remaining membranes. Locate the hindlimb buds.

NOTE: Limb buds are rounded structures situated along the anterior-posterior axis of the embryo as protrusions from the flank. The hindlimbs are located more posteriorly than the forelimbs.

2.6. Maintain the embryos in clean 1x PBS, and using a pair of fine surgical forceps, snip each hindlimb bud longitudinally. Cut the bud very close to the flank of the embryo to dissect out the whole hindlimb buds. Repeat this step with the remaining embryos.

2.7. Use a plastic transfer pipette to transfer the limb buds into an empty 1.5 mL microcentrifuge tube.

2.8. Use a pipette tip to remove any excess of 1x PBS and replace PBS with 500 µL of 0.5% trypsin solution (see **Table of Materials**). Incubate limb buds in a thermoblock for 7 min at 37 °C.

2.9. Remove the trypsin solution and replace it with 500 µL of collagenase type IV at 2 mg/mL in Hanks Balanced Salt Solution (see **Table of Materials**), then incubate it in a thermoblock at 37 °C for 8 min.

NOTE: Trypsin incubation slightly detaches the ectoderms, while collagenase disaggregate mesodermal cells.

2.10. After incubation, remove as much collagenase as possible and replace it with 1 mL of cold Dulbecco's Modified Eagle Medium-high glucose (DMEM-HG) medium supplemented with 10%

fetal bovine serum (FBS) to inactivate the enzymes. Pipette the mixture gently ~10 times.

2.11. Filter the suspension containing the cells with a cell strainer of 70 µm to leave behind the ectoderms.

NOTE: Filtration will also remove any undigested limb buds, leaving behind a suspension of single cells.

2.12. After filtering, pipette the suspension again 5–10 times and centrifuge at 200 x *g* for 5 min at room temperature, then discard the supernatant.

2.13. Use 1 ml of DMEM-HG supplemented with 10% FBS to wash the excess collagenase. Centrifuge the suspension at 200 x *g* for 6 min at room temperature.

2.14. Discard the medium carefully by pipetting and then replace it with 1.5 mL of fresh DMEM-HG supplemented with 10% FBS without disturbing the cells in the bottom of the tube.

2.15. Allow the cells to form a compact pellet (reaggregate) after incubating them for 1-1.5 h at 37 °C in a thermoblock.

NOTE: While mesodermal cells are forming the pellet, it is convenient to obtain the limb ectoderms.

3. Obtaining the limb ectoderms

3.1. Repeat steps 2.1–2.5 separately with the other 22 HH chicken embryos chosen to obtain the ectoderms.

NOTE: The number of embryos for this purpose is proportional to the final number of the RL desired. A ratio of 2:1 ectoderms-RL is appropriate.

3.2. After obtaining the hindlimb buds, use a plastic pipette to transfer them into an empty microcentrifuge tube. Remove any excess 1x PBS and replace it with 500 µL of 0.5% trypsin in sterile 1x PBS. Incubate the mixture for 30 min at 37 °C in a thermoblock.

3.3. After the enzymatic digestion, transfer the trypsin solution containing the limb buds into a sterile Petri dish.

3.4. Remove the excess trypsin as much as possible using a micropipette; flood the Petri dish with ice-cold 1x PBS supplemented with 10% of FBS until all limb buds are covered.

NOTE: FBS can be substituted for horse serum.

3.5. Identify the limb buds under the stereomicroscope; identify the ectoderm as a slightly

detached transparent membrane from the limb mesodermal cells (**Figure 1B**).

3.6. Hold the most proximal end of the limb bud with the aid of fine surgical forceps while carefully detach and separate the ectoderm layer using the other forceps.

NOTE: Obtaining the ectoderms could be difficult due to mesodermal cell clumping and attaching to ectoderms, thus causing them to become sticky. It is recommended to constantly discard the mesodermal cells to maintain only the ectoderms in the Petri dish.

3.7. Maintain the ectoderms in ice-cold 1x PBS-10% FBS solution.

NOTE: Ectoderms can be stored at 4 °C in case the reaggregated mesoderm is not ready. However, it is preferable to coordinate the incubation time of the pellet with the ectodermal obtaining.

4. Assembling mesodermal cells inside the ectodermal hull

NOTE: For this, it is necessary to have the empty ectoderms in a Petri dish with sterile 1x PBS-10% FBS solution containing a formed pellet of mesodermal cells.

4.1. After the pellet is formed (step 2.1-2.12), discard ~600 µL of the medium from the tube by pipetting.

4.2. Carefully detach the pellet from the bottom using a pipette tip and without smashing or destroying it.

4.3. When the pellet is completely detached from the bottom of the tube, turn the tube upside down to transfer the pellet into the Petri dish containing the empty ectoderms (**Figure 1C**).

4.4. Cut a small piece of the pellet with the aid of a pair of fine surgical forceps and place it as close as possible to the ectoderm.

4.5. As if it were a bag, open the ectoderm with the surgical forceps, and place the piece of pellet as tightly as possible into the ectodermal hull. Repeat this step for as many RLs as desired (**Figure 1D**).

NOTE: Cut the pieces of the pellet one by one, and fill the ectoderms to maintain clean the 1x PBS-10% FBS solution. The size of the pellet needs to correspond to each ectoderm.

4.6. Allow the ectoderm and the mesoderm to heal together for ~30 min at room temperature and then graf them into the embryo host. Discard the unused ectoderm and mesoderm.

5. Transplantation of the filled ectoderm into a host embryo

5.1. Select the number of desired eggs to graft the filled ectoderms.

5.2. Using the end of a blunt forceps, tap the eggshell of the 22 HH host embryos to open a window. Identify the air membrane and, using a pair of fine surgical forceps, remove it completely.

5.3. Open the amniotic sac near the forelimb to expose the right flank of the embryo. Open only the amount needed to accomplish the procedure.

5.4. Guided by the forelimb position, perform wound scratching with a tungsten needle (see **Table of Materials**) to the length of 2-3 somites, slightly damaging the mesoderm until it bleeds.

5.5. Individually transfer the RL into the chick embryo and place the base of the recombinant limb over the somite wound.

NOTE: RL transfer can be done with a plastic pipette or with an angled slit knife.

5.6. Fix the RL correctly with two pieces of palladium wire of 0.025 mm diameter and 0.5–1 mm length (see **Table of Materials**). Align the base of the RL with the wound to ensure that it will be attached to the flank of the host embryo and vascularized (**Figure 1E**).

5.7. Seal the window with tape, and return the egg to the incubator. Collect the embryos at the desired time points for analysis.

REPRESENTATIVE RESULTS:

Recognizing a well-performed recombinant limb

After grafting, the manipulated embryos were returned to the incubator to allow the RL to develop. The incubation time correlated with the requirements of the experiment. Nevertheless, the RL can be easily distinguished after 12 h of implantation. To determine whether the implantation was adequate, the RL was observed as a protuberance that was securely attached to the mesodermal wall of the donor embryo (**Figure 2A**). On the contrary, whether either cell viability and/or the graft failed, the RL was detached from the mesodermal wall or presented a rough morphology (**Figure 2B**).

Morphological and patterning examination of recombinant limbs

For morphological examination, RL was stained with Alcian blue[17] to observe the formation of skeletal elements and their patterning. It is recommended to stain the whole trunk of the donor embryo to avoid missing the RL during the procedure (**Figure 3A**). Alternatively, before clearing the Alcian blue, images in ethanol solution were obtained to observe the morphology of the RL or perform quantitative measurements (**Figure 3B**). Stained or unstained RL was sliced to observe tissue structure or identify cell type (**Figure 3C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of the experimental design of recombinant limbs (RL). (**A**) RL was performed by assembling limb bud mesoderm from a donor 22 HH chick embryo inside an ectodermal hull obtained from another 22 HH chick embryo donor. Ectoderms were tightly stuffed with mesodermal cells. After assembly, stuffed ectoderms were transferred and fixed with palladium wires on top of a previous somite's wound. (**B**) A limb bud with its ectoderm detached after trypsin treatment. (**C**) The pellet was obtained after its formation near to the empty ectoderms, ready to be filled. (**D**) An ectodermal jacket filled with mesodermal cells is shown. (**E**) Fixing the RL in the host embryo with palladium wires. Please note that the RL was positioned on the embryo's right flank near the forelimb bud; p: pellet. Scale bar = 500 µm.

Figure 2: Freshly obtained chicken-chicken recombinant limbs. (**A**) A 24 h RL attached to the mesoderm wall is shown. (**B**) An unsuccessful 24 h RL is shown. Please note that the palladium wire is not fixing the RL; consequently, the RL detached from the mesodermal wall and presented a rough morphology. Scale bar = 500 µm.

Figure 3: Morphological analysis of recombinant limbs. (**A**) Alcian blue staining to demonstrate skeletal elements in a 6-day chicken–chicken RL. (**B**) The same RL was shown in (**A**) before clearing the Alcian blue. (**C**) Sagittal slice of an RL stained with Alcian blue staining and with hematoxylin and eosin. Scale bar = 100 µm.

DISCUSSION:

In general, the RL protocol can be divided into five steps: (1) embryo incubation, (2) obtaining limb mesodermal cells to fill the ectoderms, (3) obtaining the ectoderms, (4) assembling mesodermal cells inside the ectodermal hulls, and (5) transplantation of the filled ectoderms into the host embryos. The major limitation of the RL technique is the long, detailed protocol, which has many critical points that require patience to perform appropriately. To successfully complete the protocol, critical moments need to be identified. During mesodermal cell procurement, the integrity and viability of the cells are essential. Cell death will prevent proper RL development. In a similar vein, correct ectoderm manipulation is necessary to guarantee the interaction between mesodermal and ectodermal cells. When ectoderms are stuffed, mesodermal cells must be as close as possible to the distal ectoderm beneath the AER. For both mesodermal and ectodermal procurement, the developmental stage of the donor embryos is also critical. It must be considered that the mesodermal cells will respond differentially according to their developmental stage. However, the developing stage can be freely selected according to the experimental requirements. Still, the 22 HH stage needs to be maintained to make obtaining ectoderms easier, thus maintaining cellular integrity and signaling. Finally, good grafting and fixing are essential for ensuring correct RL integration to the embryo wall and its development.

Edgar Zwilling first reported the RL system in 1964[6], after which many research groups implemented it to answer several interesting biological questions. The protocol of the RL has been previously described in length by Marian Ros et al. as a standard method to manipulate the developing chick limb bud[18], which explains other ways to window the eggs and perform RL with whole wing or leg and to perform RL with reaggregated mesoderm. However, some variations

between the Ros. et al. protocol and the present protocol described here can be found. In their protocol, limb buds from embryos as ectoderm donors are incubated with trypsin in ice-cold PBS for ~2 h. After initiating ectoderm incubation, they immediately obtained limb buds from embryos as mesoderm donors, then fragmented the limb buds, digested them, and removed the ectoderm manually to form the mesodermal pellet after incubating for 30 min. Here, first, the limb buds were obtained from embryos to be used as mesodermal donors, after which the whole limb bud is digested by incubating with trypsin and collagenase. Ectoderms are then removed by filtration, and the pellet is incubated between 1-1.5 h. The advantage of this method to obtain the mesodermal pellet is that the treatment with trypsin detaches the intact ectoderms from the mesoderm while the collagenase treatment digests mesodermal cells. Therefore, it is possible to filter the ectodermal tissue and discard it. On the other hand, more pellet incubation time allows it to compact better, which helps when ectoderms fill. Another difference between the two protocols is that Ros et al. peeled off the ectoderms one by one and transferred them to the Petri dish containing the pellet. In contrast, all the ectoderms are dissected and collected in a Petri dish in the present protocol. The pellet is transferred to the Petri dish to fill the ectoderms. By following this method, the ectoderms can be prepared simultaneously with grafting. As with many other protocols, how RLs are performed may vary; however, the steps in both protocols adequately describe the critical stages of the technique to produce a successful manipulation.

Previous work has demonstrated that dissociated polarizing zone cells inhibit morphogenesis when randomly dispersed among mesoderm in RL[7,10]. Thus, it is optional to eliminate cells from the ZPA before forming the mesodermal pellet. Later, the ZPA cells (or sonic hedgehog embedded beads) can be used to induce the RL to develop A-P polarity[8,9,19].

The RL experimental model is adaptable to a variety of scenarios. Recombination can be implemented with limb cells from different developmental or mature (fore- or hindlimb) stages, other positions along the three limb axes, and with dissociated–reaggregated cells or undissociated-fragmented mesoderm[15]. Interestingly, previous studies have reported using the RL model to study the behavior of different combinations of mutant and wild-type mesoderm and ectoderms[13,14,20,21] or using electroporated cells[22].

Considering that limb development is an evolutionarily conserved process, the ectoderm sources also can vary from the chicken, quail, duck, mouse, or rat ectoderms can be obtained following the same described protocol. Another possibility is changing the mesodermal -or even other cell types or sources to produce interspecies RL.

In conclusion, RL is a phenomenal model to study morphogenesis, patterning, cell-cell interactions, cell migration, and cell differentiation at the cellular and molecular levels. Because the procedure of RL allows multiple variations, it permits potential applications across numerous biological questions without being restricted to chicken-limb developmental biology.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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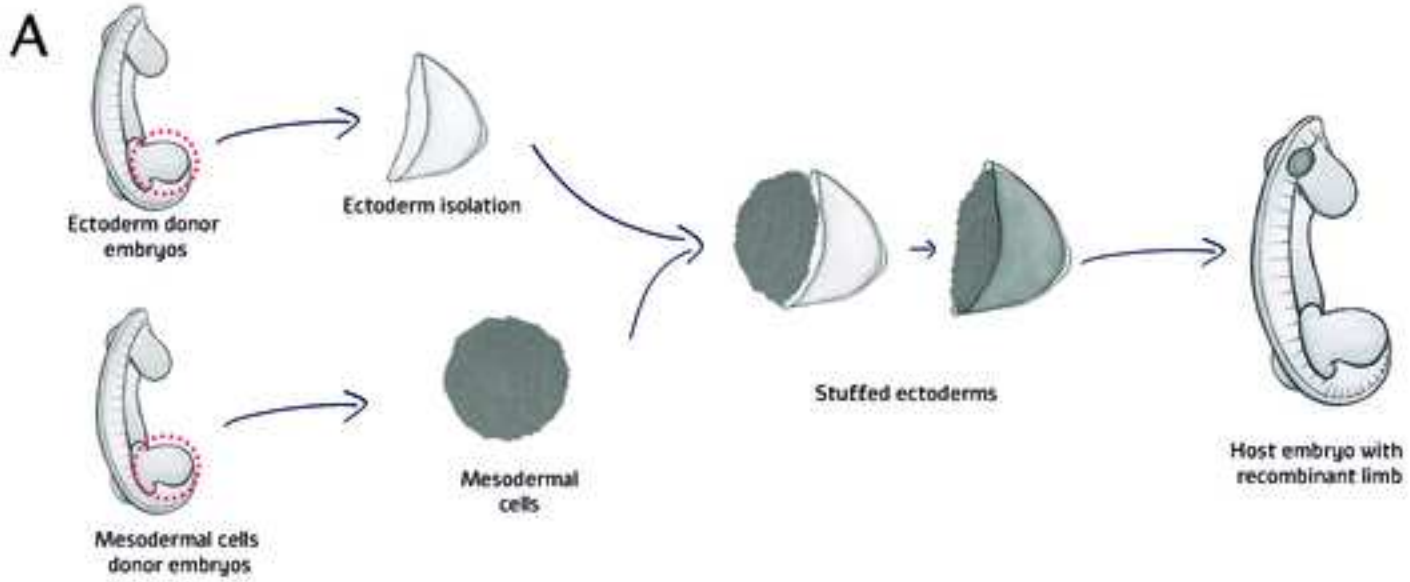

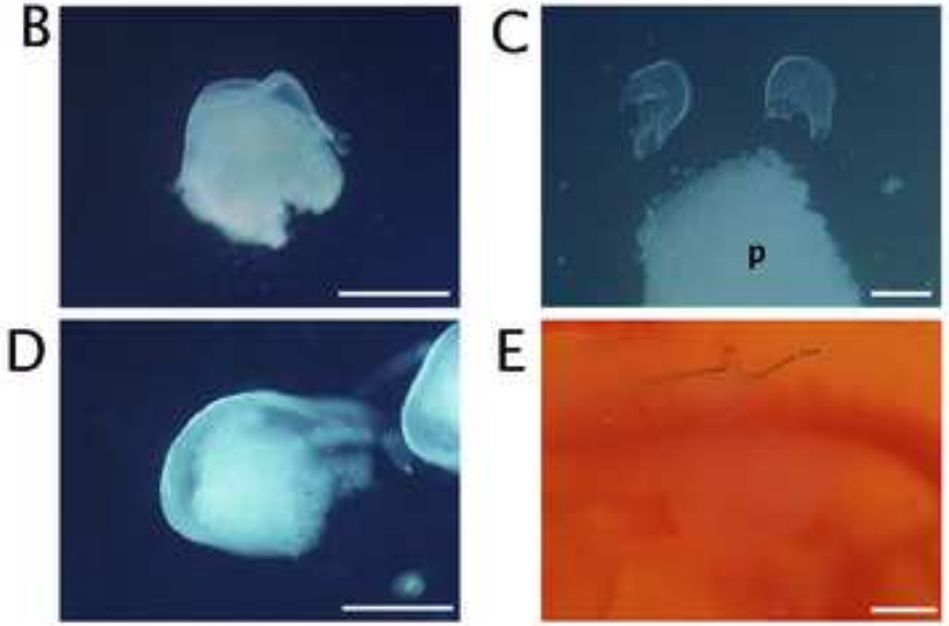

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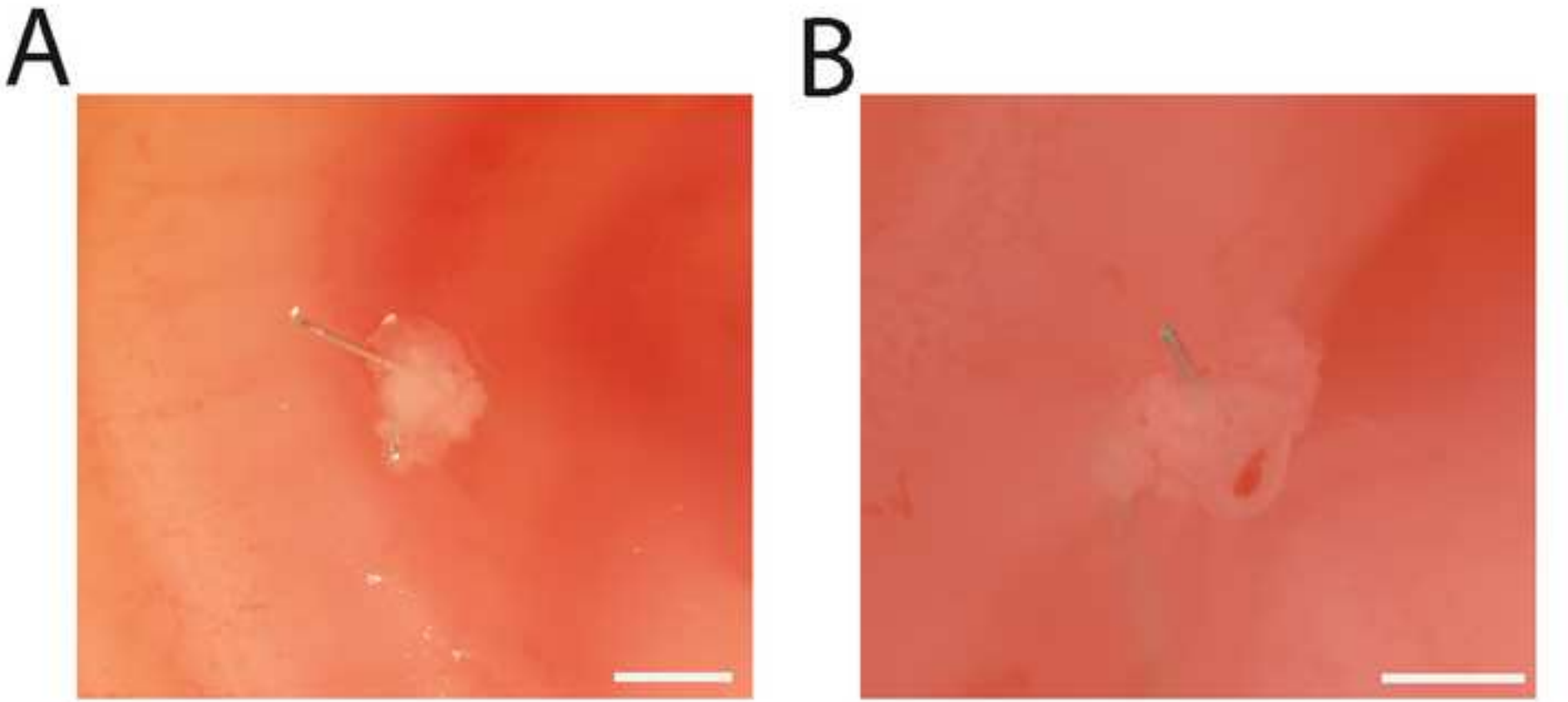

Figure 3

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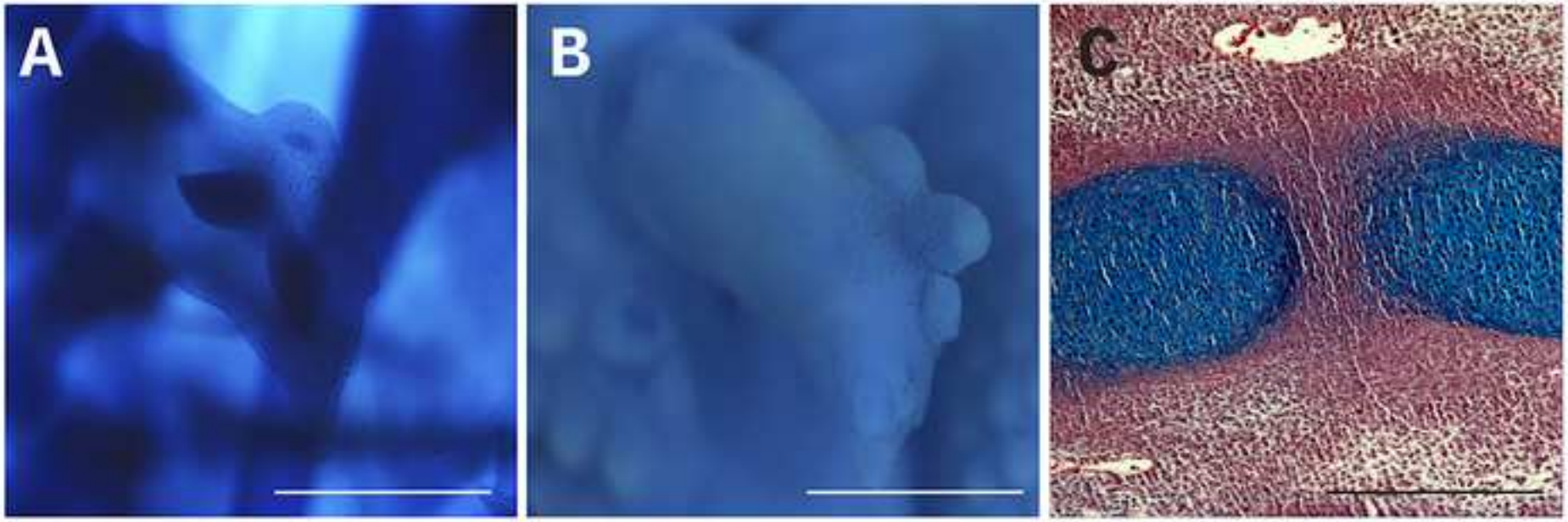

Table of Materials

Please consider our revised manuscript entitled "Chicken recombinant limbs assay as a powerful tool to understand morphogenesis, patterning, and early steps in cell differentiation" by Jessica Cristina Marín-Llera, Montse Fernandez-Calderon and Jesús Chimal-Monroy for publication in the Journal of Visualized Experiments (JoVE). We appreciate the work and criticisms that the Editorial and Reviewers put into the constructive revision of the manuscript. We incorporated all changes the Editorial and Reviewers suggested, highlighted in the manuscript, and included in this response letter. We hope that you find this revised version suitable for publication in JoVE.

Editorial comments:

Editorial Changes
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Authors: Thank you for the observations. We revised the manuscript and corrected the grammar issues.

2. Please also include the following in the Introduction along with appropriate citations:
a) The advantages over alternative techniques with applicable references to previous studies
b) A description of the context of the technique in the wider body of literature
c) Information to help readers to determine whether the method is appropriate for their application

Authors: We included in the Introduction the suggested points. The new text is highlighted in green

3. Please define all abbreviations upon first use. For example, ZPA, etc.

Authors: We revised that all abbreviations are defined. Please see the text highlighted in green.

4. Please use x g for centrifugation speed.

Authors: We confirmed that centrifugation speed is expressed in g.

5. The manuscript language is not in accordance with publication standards. Please employ professional copyediting services to improve the readability of your manuscript.

Authors: We employed professional copyediting services in this version of the manuscript.

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Readers of all levels of experience and expertise should be able to follow your protocol.

Step 1.2, 5.1: Do you disinfect the area before opening the window? If yes, please describe how it is done, also mentioning the solution(s) is used for disinfecting, round of disinfection, etc.

Authors: We included this information in the protocol.

Step 2.1: How was the cell membrane identified and discarded? How was the amniotic membrane opened? Please describe all the steps associated.
Step 2.3: How was the limb bud located? How was bud snipped? How were the whole hindlimb buds identified, isolated, and dissected? Please provide all the steps required to perform this action item.
Step 2.5 NOTE: What was the size of the cell strainer used?
Step 2.6: Should be "without disturbing" instead of resuspend". Please check and correct.
Step 2.8: Was the DMEM medium removed before the cells were left to form a pellet? If yes, please mention this before this step. Also, was any medium or any other solution added to the cells to help form a compact pellet? If yes, please mention what solution was used.
Step 3.2 NOTE: The steps mentioned are a bit confusing. Do you mean that: The buds are transferred to an empty tube, followed by PBS addition? The PBS is then removed and then trypsin is added? Please clarify.
Step 3.5: How was this done? Please provide all the steps including how were the mesodermal cells removed.
Step 3.6: Should be FBS not SFB. Please correct.
Line 134, 174: NOTE 2 is redundant and can be discussed in the protocol Discussion as a modification of the protocol.
Step 5.1: How were the 15-20 somites located in the embryo?
Step 5.2: How was the wounding done? How was it ensured that the mesoderm is damaged? Please describe.
Step 5.4: How was RL alignment with the wound ensured?

Authors: Thank you. We considered all your comments in the protocol and provided more details in each step.

7. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Authors: We revised the "notes" and included some of them as steps in the protocol. Redundant notes were eliminated.

8. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Authors: Essential steps are highlighted in yellow.

9. For all figures, please provide the panel labels outside of the image/figure.
10. Figure 1B-F, 2: Are these images taken through a microscope? If yes, please provide an appropriate scale bar to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Authors: We modified the figures and figure legends according to your comments.

11. Figure 1B-D: What does the p in the images stand for?

Authors: The letter p indicates "pellet". The meaning of p is included in the legend of Figure 1.

12. Please also include the following in the Discussion along with appropriate citations:
a) Critical steps within the protocol
b) Any limitations of the technique

Authors: We included in the discussion the suggested points. The text is highlighted in green

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript describes a method for producing recombinant limb buds using chicken embryos. The recombinant limb bud assay helps study the interaction between mesenchyme and ectoderm in limb development. This manuscript provides the reader with a method for approaching critical questions in limb development and embryogenesis.

Major Concerns:
Overall, there are few problems with the accuracy of the text, but the quality of the figures is quite poor. In particular, Fig. 1B-F, Fig. 2A, B, and Fig. 3A, B, the color production and brightness/contrast adjustments are so bad that the objects are entirely unrecognizable. The authors need to revise these figures completely.

Authors: We appreciate your observation. We revised and improved all the figures. Please see the new figures.

Minor Concerns:
Line 216, The meaning of "The meaning the RL will develop until their collection" is unclear. It should be described in detail so that readers can follow its meaning.

Authors: Thank you for the observation. We clarified this point in the text; please see the text highlighted in green.

Reviewer #2:
Manuscript Summary:
Marin-Llera et al. present a working protocol to perform the powerful recombinant limb technique using chicken limb buds. The description is easy to follow, but I think the video will be much more important than the text for some of the steps. The discussion about the potential uses of this technique is thoughtful and to the point. However, there are some concerns that need to be addressed.

Major Concerns:
- This technique is a classic, and was very successfully reestablished by John Fallon and his scientific lineage. In particular Marian Ros's lab has published several papers describing the protocol at length (see chapter 25 of Methods in Molecular Biology, Vol. 137 (year 2000): Developmental Biology Protocols, Vol. III). While the authors are of course entitled to publish their own version of the protocol, they should refer to the mentioned book chapter and explain the main differences, as the Ros protocol is arguably the standard in the field. Key differences I have found: 1) prior to mesoderm dissociation, it is recommended to remove the ectoderm, using trypsin 0.5%, 9 min at 37°C; 2) empirically, horse serum seems to work better than fetal bovine serum to keep chick cells happy after collagenase; 3) to obtain the ectoderm hulls (step 3.2), trypsin incubation is milder if done on ice for 1.5-2h. If this is done the first thing, it gives just about enough time to obtain the mesoderm pellet. Again, it's fine to present a different protocol, but the differences should be justified and discussed.

Authors: We appreciate your comment; this information is included in the discussion section. Please see the text highlighted in green.

- If the authors have done it, it would be good to show examples of inter-species recombinant limbs, as this is mentioned in the abstract as a big advantage.

Authors: We agree with your comment. However, data from interspecies RL that we have is included in another manuscript that is under review at this moment.

Minor Concerns:
- The in ovo pictures of Fig. 2 are not very useful, as the contrast is very low. I suggest to use ex ovo pictures, so that all the structures are easily discernible. Pictures in Fig. 1 could also be improved. Why do they have a pink hue?

Authors: We appreciate your observation. We revised and improved all the figures. Please see the new figures.

- Collagenase is not inactivated by serum. The most effective way of inactivating collagenase is to use low temperature and wash it extensively. I suggest the authors change the wording accordingly.

Authors: Thank you for the observation. We included that the solution needs to be cold to inactivate the collagenase.

- Some steps are taken for granted (such as removing the embryo from the egg). It would be good to show it in the video, as unexperienced researchers often struggle with this.

Authors: We agree with the reviewer. We revised the protocol and improved the explanation, and mentioned more details in all the steps.

- There are multiple grammar errors (e.g. windowed instead of windowing, mismatch between subject and verb in the sentence, etc). Also, some examples of SFB (Spanish acronym) instead of FBS.

Authors: We employed professional copyediting services in this version of the manuscript.