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Analysis of cell differentiation, morphogenesis, and patterning during chicken embryogenesis using the soaked-bead assay

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TITLE:

Analysis of Cell Differentiation, Morphogenesis, and Patterning during Chicken Embryogenesis using the Soaked-Bead Assay

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KEYWORDS:

chicken embryo, limb development, cell differentiation, morphogenesis, pattern formation, chemical/protein delivery.

SUMMARY:

The soaked bead assay involves targeted delivery of test reagent at any developmental time point to study the regulation of cell differentiation and morphogenesis. A detailed protocol, applicable to any experimental animal model, for preparing three different types of soaked beads and implanting these in the interdigit of a chicken embryo is presented.

ABSTRACT:

A multitude of genetic programs is activated during embryonic development that orchestrates cell differentiation to generate an astounding diversity of somatic cells, tissues, and organs. The precise activation of these genetic programs is regulated by morphogens, diffusible molecules that direct cell fate at different thresholds. Understanding how genetic activation coordinates morphogenesis requires the study of local interactions triggered by morphogens during development. The use of beads soaked in proteins or drugs implanted into distinct regions of the embryo enables studying the role of specific molecules in the establishment of digits and other developmental processes. This experimental technique provides information on the control of cell induction, cell fate, and pattern formation. Thus, this soaked bead assay is an extremely powerful and valuable experimental tool applicable to other embryonic models.

INTRODUCTION:

Breakthroughs in the molecular mechanisms that control gene expression during embryonic development have allowed us to understand how cell fate is determined. Commitment to

different cell lineages occurs once cells begin the molecular expression of transcription factors[1]. This expression pattern is highly coordinated in space and time and thereby directs the shaping, positioning, and patterning of cells, tissues, and organs[1–5]. Embryonic induction is the process by which cells are committed to specific lineages by establishing hierarchies that restrict cells' potentiality, which even include the generation of the basic body plan as occurs with the Spemann organizer[6,7]. The blastopore dorsal lip induces a second embryonic axis in a host embryo[8,9]. Today, with the aid of grafting and other classical experiments combined with molecular approaches, it is known that different transcription factors and growth factors function to direct embryonic induction in the Spemann organizer[10]. Thus, experimental manipulation is an important tool to understand cell differentiation, morphogenesis, and patterning processes during embryogenesis.

Interestingly, in embryonic systems where tissue transplantation is difficult or when the inducers are already well known, carriers are used to deliver molecules (e.g., proteins, chemicals, toxins, etc.) to regulate cell differentiation, morphogenesis, and even patterning. One such carrier system involves implanting beads soaked in a specific molecule in any experimental model organism at any developmental time point to determine the effect of the said reagent or direct the differentiation of the said model. For example, by implanting retinoic acid (RA)-soaked beads into the chicken wing limb bud, Cheryl Tickle et al. (1985) demonstrated that RA induces the expression of sonic hedgehog in the zone of polarizing activity (ZPA)[11,12]. The same experimental strategy was used to discover that RA controls the asymmetry of somites and cell death in the limb bud during digit development and in other embryonic limb regions[13–15]. Other factors, mainly proteins (e.g., fibroblast growth factors [FGF], transforming growth factor-beta [TGF-ß]) have been used to induce limbs in early embryos' flanks and new digits in the interdigital region, respectively[16–21]. These experiments evidence the power and utility of this technique for determining the stage of commitment or competence of tissues or groups of cells exposed to the molecules.

In this protocol, the chick limb at the stage of digit formation served as the experimental model to present step-by-step how to prepare and implant the soaked beads. However, this experimental tool is not limited to this application but can be exploited in any experimental animal model and any timepoint *in vitro* and *in vivo* to study induction, differentiation, cell death, and patterning.

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).

1. Egg incubation and embryo staging

NOTE: Fertilized hen eggs can be obtained from local farms. Fertilized White Leghorn chicken eggs are most commonly used. Store the freshly fertilized chicken eggs at 15 °C for up to 1 week

prior to incubation.

1.1. Incubate the fertilized chicken eggs vertically with the pointy side down in a humidified incubator at 38 °C and 70% relative humidity until they reach the 28 HH stage (approximately 5.5 days according to Hamilton and Hamburger, 1951)[22]. Rotate the eggs during the incubation to prevent the embryo from adhering to the shell membrane.

NOTE: The choice of the developmental stage is informed by the experimental aims. In this case, the 28 HH stage is optimal for interdigit bead implantation to induce an ectopic digit or promote cell death.

1.2. Remove the eggs from the incubator, swab them with 70% ethanol, and allow them to air dry. Disinfect the working area, microscopes, and instruments with 70% ethanol.

1.3. Candle the egg to identify blood vessels and locate the embryo. Discard eggs that do not have an embryo.

1.4. Using the end of non-toothed forceps, open a window by tapping the blunt end of an egg, and remove a 1-cm^2 section of the shell with the forceps.

1.5. Transfer the egg into a carton or plastic holder and place it under the stereomicroscope. Remove the air membrane by puncturing and pulling it out with fine surgical forceps. Remove any small piece of eggshell that could contact the embryo.

NOTE: The air membrane is the white, opaque membrane observed immediately after windowing the egg.

1.6. Observe under the microscope while opening the amniotic sac slightly, tearing it using the fine surgical forceps. Be careful not to damage the vasculature of the chorioallantoic membrane.

NOTE: The amnion is the transparent membrane closely surrounding the embryo that encapsulates it in amniotic fluid.

1.7. Stage the embryos *in ovo* to determine whether they are in the desired stage. Embryos in earlier stages can be returned to the incubator after sealing the eggshell window with tape.

2. Bead preparation

NOTE: Depending on the experimental aim and treatment in question, alternative bead types (e.g., Affi-Gel, AG1-X2, heparin) may be more suitable. Affi-Gel beads are optimal for proteins (e.g., TGF-ß1), while heparin beads are ideal for growth factors (e.g., FGFs, WNT) and AG1-X2 beads for chemicals solubilized in organic solvents (e.g., DMSO).

2.1. Affi-Gel and heparin bead preparation

2.1.1. Cut a square of parafilm to fit a 45-mm Petri dish. Place the parafilm across the bottom of the petri dish to cover it and fix it to the bottom of the Petri dish by pushing each vertex using the end of non-toothed forceps. Set aside.

2.1.2. Using a pipette or spatula, transfer the beads into a microcentrifuge tube and wash them twice in 1x PBS by settling and pipetting.

2.1.3. Transfer ~40–50 beads with a micropipette to the center of the parafilmed Petri dish from step 2.1.1.

2.1.4. Using the microscope, select ~30 Affi-Gel or heparin beads ~100 µm in diameter for use. Use a microscope eyepiece reticle to size the beads or use the third interdigit of an HH 28 embryo as a reference; the bead must be smaller than the interdigit.

2.1.5. Carefully remove as much of the excess PBS surrounding the beads as possible and soak them in 2–5 µL of the treatment solution. Assure that the solution completely covers beads.

2.1.6. In parallel, prepare control beads by soaking in a solution that contains the same amount of vehicle as used in the experimental treatment solution.

NOTE: Concentrations need to be calculated for each treatment according to the experiment. Use the appropriate personal protective equipment when handling potentially harmful reagents.

2.1.7. Incubate the beads in the solution for 30 min at room temperature. To prevent the beads from drying out during the incubation, pipette a few drops of 1xPBS or water around the beads to humidify the local atmosphere and cover the dish with parafilm to slow evaporation.

2.1.8. Place the Petri dish on ice and implant the beads within the same day.

2.2. AG 1-X2 bead preparation

2.2.1. Cut a square of parafilm to fit a 45-mm Petri dish. Cover the bottom of the Petri dish with parafilm and affix by pushing each vertex using the end of non-toothed forceps. Set aside.

2.2.2. Use a spatula to transfer the AG 1-X2 beads into a microcentrifuge tube. Add 50 µL of the treatment solution at the desired final concentration.

2.2.3. In parallel, incubate the control beads in a solution with the vehicle alone, prepared without the experimental chemical or protein of interest.

2.2.4. Incubate the beads for 20 min while slowly shaking at room temperature. Wrap the microcentrifuge tubes with foil to protect from light during all incubation, given that many of these molecules are light-sensitive

2.2.5. Remove as much of the solution as possible using a pipette and stain with 2% phenol red dissolved in water at room temperature for 2 min with mild agitation in a vortex.

NOTE: Dyeing the transparent AG 1-X2 beads facilitates their implantation in the embryo.

2.2.6. Remove the 2% phenol red and wash the beads twice in 1x PBS to remove any excess dye.

2.2.7. With a micropipette tip, transfer ~40–50 AG 1-X2 beads to the center of the parafilm-covered Petri dish prepared in step 2.2.1 and soak these in 5 µL of 1x PBS. Under the microscope, select around 30 beads that are ~100 µm diameter in size. Discard the unused beads.

2.2.8. Ensure the beads are submerged in the experimental solution throughout the incubation by pipetting a few drops of 1x PBS or water around the beads to humidify the local atmosphere and/or cover the dish with parafilm to slow evaporation.

3. Embryo manipulation and interdigit implantation

3.1. Before manipulating the embryos, arrange two stereomicroscopes next to each other on a benchtop. One is for embryo manipulation and bead implantation, and the other is for maintaining the treated beads ready to implant into the embryo.

3.2. Using non-toothed forceps, create a window in the remaining eggs as described in steps 1.4 and 1.5.

3.3. Under the microscope, open the amniotic sac by tearing the amniotic membrane with fine surgical forceps near the right hindlimb only by the amount needed to accomplish the procedure (i.e., as little as possible).

3.4. Using fine forceps, hold the embryo by the amniotic membrane to expose the right hindlimb. Using a fine tungsten needle, make a hole centered in the distal-most of the third interdigit of the hindlimb.

3.5. Without releasing the embryo and the exposed hindlimb, take one treated bead from the other microscope using one of the tips of the forceps. The forceps must be in the open position for one bead to adhere to the forceps tip.

3.6. Transfer the bead into the chick embryo near the hindlimb and position it on top of the interdigit hole.

3.7. Close the forceps to apply pressure to the bead until it enters the hole.

3.8. Release the embryo and seal the eggshell window with tape.

3.9. Return the eggs to the incubator until they have reached the required stage. Repeat the procedure until the required number of manipulated embryos is reached. It is imperative to ensure that the beads do not dry out at any point.

NOTE: To observe a complete ectopic finger, incubate for ~72 h after bead implantation. In contrast, early differentiation genes (e.g., *Sox9*) are expressed ~30 min after the implantation of a TGF-ß1-soaked bead.

REPRESENTATIVE RESULTS:

Using soaked beads to evaluate cell behavior in the embryonic chick limb

To assure the efficacy of this assay, the bead must be placed consistently and precisely in the correct location; in this case, the distal-most of the third interdigit beneath the apical ectodermal ridge AER (**Figure 1A**). This positioning permits the molecule in question to spread equally throughout the interdigital tissue. Moreover, the zone beneath the AER contains undifferentiated cells that are readily responsive to treatment. To evaluate the effects on cell differentiation, the embryos can be stained with Alcian blue and Alizarin red to evidence the formation of skeletal elements (**Figure 1B**). The soaked-bead assay is also well-suited for evaluating cell death with neutral red (**Figure 1C**) and gene regulation by *in situ* hybridization (**Figure 1D**). Scale bar is set at 250 µm.

FIGURE AND TABLE LEGENDS:

Figure 1: Bead implantation into the interdigital tissue of the chick limb. (**A**) The correct location for the soaked bead beneath the AER in a 28 HH hindlimb**.** (**B**) Alcian blue and Alizarin red skeletal staining evidence the formation of an ectopic digit induced 4 days after implanting a TGF-ß1-soaked bead. (**C**) Neutral red staining marks cell death induction (arrowhead) 24 h after implanting a RA-soaked bead into the interdigital tissue at the 28 HH stage. (**D**) Sox9 *in situ* hybridization 4 h after a TGF-ß1-soaked bead was implanted. Scale bar is set at 250 µm. The images shown in **B** and **C** were taken from Díaz-Hernández et al.[23,24].

DISCUSSION:

The main advantage of the experimental tool detailed in this protocol is being able to control the time and location of the exposure to beads soaked in a given experimental molecule. Combining the correct positioning with precise developmental timing provides enormous possibilities to study cell differentiation processes. Performing these experiments in undifferentiated tissue enables investigating the first crucial events in cellular lineage. For example, placing a TGFß-soaked bead in the interdigital tissue of embryonic limbs 28 HH results in the formation of an ectopic digit in which a molecular cascade is triggered that induces the genetic expression of the master gen *Sox9*[25]. Remarkably, the induced cartilage tissue also organizes into a digit with phalanx formation.

Interestingly, RA triggers cell death in the same interdigital region by regulating the gene expression of bone morphogenetic proteins that direct the cell fate of undifferentiated cells toward cell death[10]. Hence, cell differentiation, cell death, morphogenesis, and patterning can be concurrently investigated in the same region of an embryo and tailored to any region and genetic

pathway of interest[8,9].

The elements crucial to the success of this protocol include never letting the soaked beads dry out (i.e., they must always remain wet). Also, selecting the appropriate beads is essential: Affi-Gel and heparin beads are for proteins, whereas AG1-X2 are for chemicals dissolved in organic solvents. Another critical point is the concentration of the molecule contained in the solution used to soak the beads, which is usually 1000-fold more concentrated than would be used for *in vitro* studies. Nevertheless, an inconvenience of this method is that the final concentration of molecules released from the soaked beads is unknown, as well as the velocity of release. In the protocol is mentioned that beads-100 µm diameter is more convenient for use. Consider this when limbs are manipulated. Most importantly, the diameter of the beads must be selected according to the implantation zone and consistently maintained in each embryo. However, a slight variation in bead size between experiments is not likely to affect the results.

In conclusion, the potential of the soaked-bead assay outlined here depends only on the imagination of the researcher. This protocol can be applied to any experimental animal, cell culture, or organotypic culture model, including organoids. Furthermore, this protocol is a helpful, straightforward educational tool for teaching students basic developmental biology concepts and technical skills by practicing this experimental manipulation in developmental biology classes.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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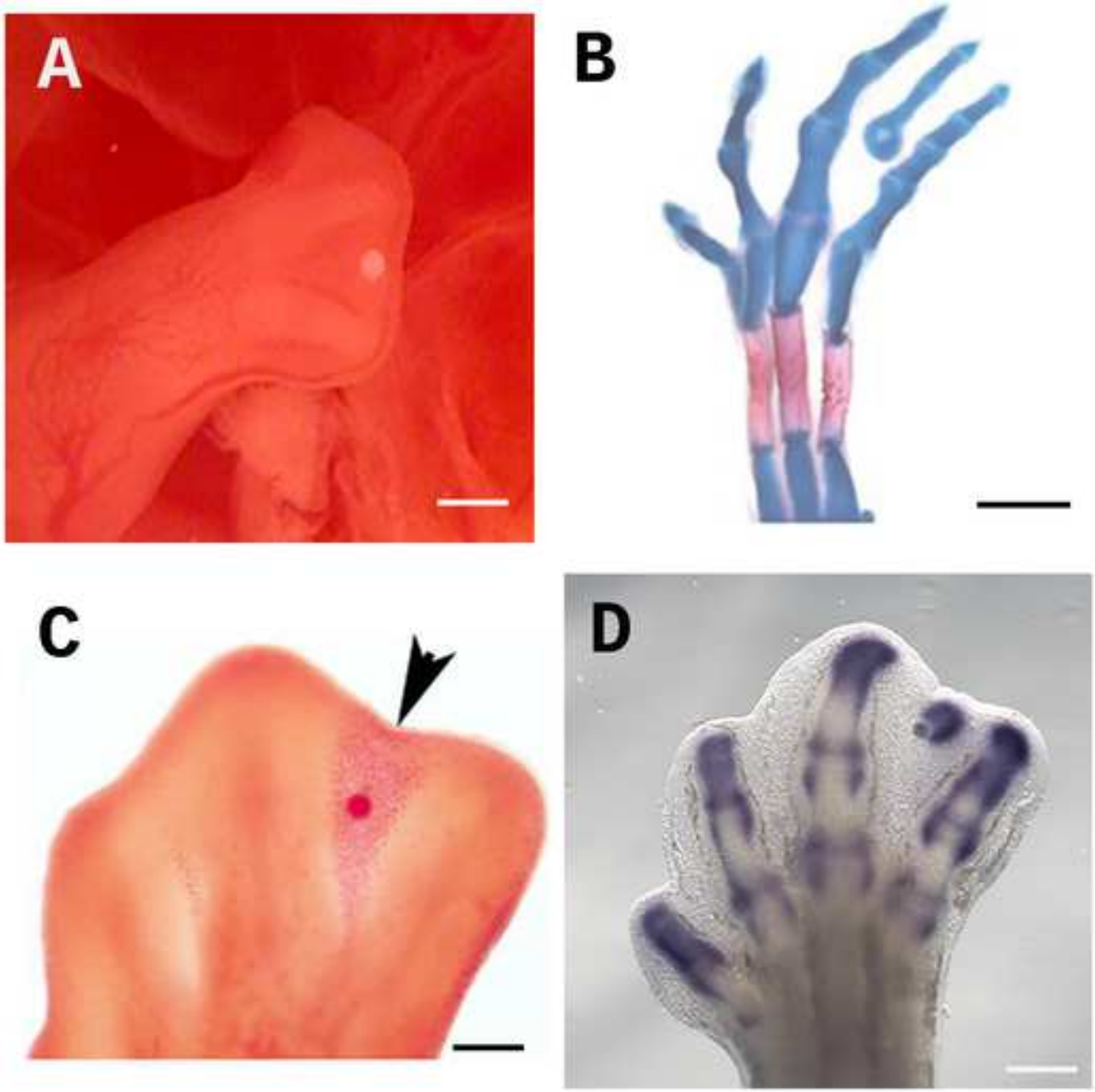

Editorial comments:

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

Authors: We used copyediting services to revise the grammar and spelling issues in the manuscript.

2. Please provide at least 6 keywords or phrases.

Authors: We provided the keywords

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Authors: We revised the text to avoid these personal pronouns.

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For example: Affi-Gel, etc.

Authors: There is no commercial language in the manuscript

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Authors: We verify the protocol is written in the imperative tense. We also included the use of personal protection where is necessary.

6. Line 73: Please add “Hamilton and Hamburger, 1951” in the references and cite the corresponding reference number here.

Authors: We included it in the reference list.

7. 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 in the manuscript

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Authors: The figures presented in Figure 1B corresponded to the figure of our article published in PlosOne under the Creative Commons Attribution (CC BY) license. Reference 23 is licensed under CC BY by Jesus Chimal-Monroy. Figure 1C corresponded to figure of our article published in Journal of Developmental Biology under (CC BY) license. Reference 23 is licensed under CC BY by Jesus Chimal-Monroy

9. As we are a methods journal, please ensure that the Discussion explicitly covers 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

Authors: The Discussion section includes these points.

10. Please title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Authors: Citations are according to your comment.

11. Figure 1: Please consider adding a scale bar to the images in the panel.

Authors: Scale bars were included in the figure.

12. Please ensure that the Table of Materials includes all the essential items (reagents, chemicals, equipment, instruments, etc.) used in this study and sort the table in alphabetical order.

Authors: The table of materials includes the essential items and is sorted in alphabetical order.

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors describe how to apply beads soaked with proteins or other drugs to the chicken hind limb. The authors give a short description of what is needed and necessary for the procedure and show examples of the induction of additional digits as results. They discuss the advantages of disturbing morphogenesis at specific time points and tissue positions to be able to evaluate the effect of different concentrations of a protein/drug or the effects of different proteins/drugs.

The method can be applied to other structures, tissues and animals to study the effect of excess proteins or drugs or blocking of those on the development of cell types and or tissue morphogenesis.

I am looking forward to the video.

Major Concerns:

1. I have a question and suggestion concerning the data in the pictures. The authors show the result of two 4d treatments with TGFß1 in Figure 1B and D. The additional digit in Fig 1B looks longer than the one in 1D. The difference could be due to the concentration or the release of the TGFß1 in a certain time period, I assume. This a nice example that there can be variations in the results as the authors mention in their discussion (line 215). The authors should mention this effect as an example and discuss it. Since, it also means that the concentration of protein on the beads that spreads to the environment is not really controllable. Therefore, slight variations in the size of the beads might be not significant.

Authors: Thank you for your observation. However, the treatment shown in figure 1D is for four hours, not four days, as figure 1B shows. It is specified in the figure legend. Nevertheless, we included in the Discussion that variations in the size of beads may not affect the results. Please see the text highlighted in cyan

2. The authors describe the application of a bead on an embryonic chick hindlimb. They point out - in introduction and abstract and in their final conclusion- that the method can be used in other systems and animals, with which I agree. However, I found it a bit irritating that the publication they cite on the importance of correct patterning and morphogenesis during development is their own publication on cell fate decisions in chick limb development. The theory that different concentrations of a single molecule can instruct distinct cell types goes back to the mid of last century and there are maybe other more general review articles to cite, like Stapornwongkul, 2021, Rogers 2011, Irizarry, 2021, Capek 2019, Vargessib 2020, to name a few.

Authors: We agree with your comment. We added the references you suggested.

3. The protocol description has a lot of 'notes' and some are repetitive. This could be changed by moving the similar notes just to the end of the paragraph on how to prepare the beads. Some other notes could actually be included into the description.

Authors: We revised the notes and included them in the protocol.

Minor Concerns:

line 72-73 describes the incubation of the chick egg. I assume, the authors incubate the egg with the pointed end at the bottom and the rounder end that contains the air bubble on the top. The other possibility used with chick egg experiments is to lay the eggs on the long side without rotating them regularly. Thus, the embryo can always be found at the top of the egg. The authors

should include a more detailed describtion which part of the egg is up or down in the instruction in 1.1.

Authors: We agree with your comment. We included more details in all steps of the protocol.

line 17: '..here we presented…' might be better 'we present'

line 18: suggest ..can be used in any experimental animal model.

line 27: the authors talk about 'soaked beads in proteins or drugs' e.g. in the abstract. I suppose they mean beads soaked in proteins or drugs?

line 37: suggest how cell fate is determined.

line 51: What do the authors mean with '..Following the same..?' protocol, method, strategy??

line 73: the Hamburger & Hamilton citation is missing in the reference list.

line 96, 104, 134,136,139: ..wash twice in PBS 1x. do you mean in 1x PBS instead e.g. 10x PBS? Is 1x really necessary, if it is 1x. I would assume, if I read PBS that it is 1x PBS.

line 104: .suggest: remove as much of the excess PBS from the beads as possible. Then soak them in the…

line 118: 'place and fix parafilm in a 45 mm petri dish until cover the bottom'? I am not sure I understand that instruction.

line 126: 'Incubate in the beads…' do you mean: incubate the beads?

line 126: 'for 20 min in mild agitation..' suggest: slowly shaking

line 157: suggest: microscope using the tip of the forceps.

line 160: suggest: It is imperative to control during the entire experiment that the beads to not dry out.

line 167: '.. and return to the incubator..' whom the egg/s? ☺ suggest: … return the eggs to the incubator until they have reached the required stage/age.

line 168: 'Repeat this procedure to perform as many as desired manipulated embryos…' suggest: repeat the procedure till the required number of manipulated embryos has been reached.

line 196: suggest: ..is to be able to control time and location of the bead.

line 200: suggest:…interdigital tissue of embryonic limbs at HH28..

line 202: suggest: …organizes into a digit?

line 207: '…can be potentially studied in the same region …'. suggest: can be all studied in the same region?

line 207: 'However, placing chemical- or proteins-soaked beads in distinct embryonic regions or cell cultures depends on the interest of researchers.' do the authors mean: Which region depends on …The interest of the researcher/s can determine

line 210: soaked beads dry… suggest: not to let soaked beads dry/dry out

line 213: suggest: 1000 times higher concentrated than

line 214: suggest: an inconvenience of this method is..

line 215: suggest: unknown as well as the velocity…

line 217: ..limited to the imagination…I would put it a bit more positive, like:

depend on the imagination…

Authors: We really appreciated all your suggestions. We included all of them in the text. Please see the text highlighted in cyan.

Reviewer #2:

Manuscript Summary:

The manuscript describes in a nice detail a very well-known but rarely well described technique of beading developing chick. The description is detailed and permits anyone to reproduce it

Major Concerns:

None

Minor Concerns:

I would like to see the video before giving the final approval

Authors: We appreciate your comment. Thank you.

Reviewer #3:

Manuscript Summary:

The authors of this manuscript used the chick embryo limb as an experimental model to present step by-step preparing and implanting soaked beads into the interdigit at stage 28 (5.5 days). Authors claimed that this tool protocol can be applied in any experimental animal model in vitro and/or in vivo to study induction, differentiation, cell death, and patterning.

Major Concerns:

None

Minor Concerns:

Line 55: add the refence of Abu-Elmagd et al., 2015 since in this study beads were soaked in FGF proteins and a 2nd limb bud was successfully generated.

Line 102: Please mention here how the beads was measured individually

Line 111: Also, the dish can be covered with parafilm to avoid evaporation.

Line 129: How you do this? Is it by wrapping the dish with foil?

Line 136: Mention why you do this PBS wash before implantation? Is it to remove the excess of the soaked reagent?

Line 167: Change to: and return the operated and sealed egg to the incubator…

Authors: We really appreciated all your suggestions. We included all of them in the text. Please see the text highlighted in cyan.

-In the Table of the Materials, Sigma has discontinued Heparin beads (Cat. No. : H-5263, please have a look at their website), where authors obtained these beads from ?

Authors: Thanks for the observation. Heparin Sepharose (ab193268) from Abcam is now included in the Table of Materials.

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2. **Figure 1C** corresponded to figure of our article published in Journal of Developmental Biology under the **Creative Commons Attribution (CC BY)** license. Reference 24 is licensed under CC BY by Jesus Chimal-Monroy (**Creative Commons Attribution License**)